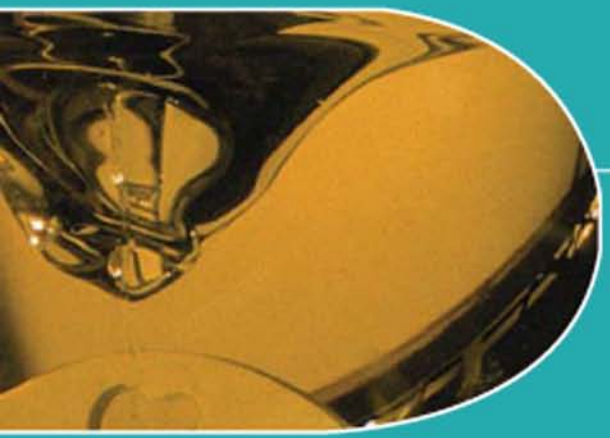

BIOEN

FAPESP

BRAZILIAN RESEARCH ON BIOENERGY

BIOENERGY: THE BRAZILIAN EXPERIENCE

Bioenergy is an important clean energy alternative. The Brazilian experience has shown that sugarcane is an extremely viable crop for the production of bioenergy.

Brazil is outstanding as the world's most intensive user of bioethanol as an alternative to gasoline for powering transport. Total bioethanol production for 2009/10 is projected at 27 billion liters in 405 plants, 157 of which are dedicated exclusively to ethanol. In 2009/10 around 57 per cent of the 600 million tons of sugarcane will be used for ethanol and 43 per cent for sugar production. The total sugarcane planted area in Brazil is around 7 million hectares (ha). This accounts for only around 2-3 per cent of total area devoted to agriculture.

CLEAN AND CHEAP ENERGY

Brazil and the USA produce 70 per cent of world's ethanol. Brazilian bioethanol production costs are the cheapest in the world. Industry estimates the cost of producing ethanol from sugarcane at approximately US$ 0.29/L (1 gallon = US$1.00).
In addition to the lower production costs, ethanol produced from sugarcane in Brazil has another important advantage: in Central-South Brazil only 1 unit of fossil energy is used for each 8-9 units of energy produced by ethanol from sugarcane. Carbon sequestration also benefits from ethanol: for each ton of ethanol used as fuel 2.3t of CO_2 are not released into atmosphere with a simultaneous reduction in SO_2 emission.

THE BRAZILIAN MODEL

Sugarcane was introduced into Brazil in 1532 by the Portuguese. Since then this crop has been gradually improved, with the development of varieties of sugarcane that enhanced the yields of both culm and sucrose. During the 1970s the "Brazilian model" of producing sugar and ethanol alongside each other, brought important technical improvements and made possible an outstanding increase in competitiveness in the international market for sugar and ethanol.

Ethanol has a great potential to become a worldwide replacement or complement for gasoline. In 2008, 75 per cent of the 3.0 million cars and light vehicles bought by Brazilians were of the flex-fuel type, which run on any proportion of ethanol and gasoline mixture. Considering the existing opportunities related to biofuels, it is expected that R&D will lead to optimization not only of the sucrose content of the plant, which is relevant for sugar production, but the overall energy content (biomass yield).

THE ENERGY-CANE

With the possibility of cellulosic ethanol production scientists are able to envisage a new option, the "energy-cane" and not only the "sugarcane", in which the whole biomass is of interest.

The development of hydrolysis and/or gasification processes could be applied to the residual bagasse and trash, transforming the lignocellulosic biomass into ethanol, using fermentation of the generated sugar (hydrolysis) or the catalytic synthesis of the generated gas (gasification). It is expected that ethanol output might increase from the current 6,000 to about 9,000 liters per hectare-year, i.e. between 40 and 50 per cent.

In Brazil in 2008, 90 per cent of the bagasse was already burned to produce energy equivalent to 1,400 MW. This is enough to meet the internal needs of the mills and the surplus energy can be fed into the country's power grid. Investments in co-generation efficiency and power distribution aim to achieve 14,000 MW of bioelectricity by 2020.

The growing demand for bioenergy brings new scientific challenges in terms of R&D and assessment of the environmental and social impacts related to the expansion of sugarcane cultivation.

Currently bioethanol is produced from fermentation of the extracted juice and the molasses resulting from the sugar industry.

But, in addition to sucrose, there is also a relevant and extractable amount of energy in the glycosidic linkages of cellulose and hemicelluloses, which account for nearly two thirds of the sugarcane plant (bagasse and leaves).

FAPESP'S BIOENERGY PROGRAM – BIOEN

To respond to the increasing need for R&D in the area of biofuel the State of São Paulo Research Foundation (FAPESP) created a Bioenergy Program (BIOEN). FAPESP is one of the Brazil´s leading public funding agencies for Scientific Research.

The FAPESP Program for Research on Bioenergy, BIOEN, aims to link public and private R&D, using academic research institutions, and industrial laboratories to advance and apply knowledge in fields related to ethanol production.

BIOEN

FAPESP

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FAPESP BIOENERGY PROGRAM

http://bioenfapesp.org

The BIOEN Program mission is to foster comprehensive academic and industry research on sugarcane and other biofuel sources integrated with the sugar and ethanol Industry, thus assuring Brazil's position among world leaders in Bioenergy research and industry.
The research agenda includes biomass production and processing, biofuel production, engines and the overall impact on land use and on socio-economics aspects.

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INNOVATIVE RESEARCH IN BIOENERGY

http://bioenfapesp.org

The BIOEN Program is built on a solid core of academic research related to biomass production and processing, biofuel production, engines and impact on land use and socio-economic matters.

It is expected that these activities will be translated into new knowledge and the training of scientists and professionals to advance industry in ethanol related technologies.

Moreover, the BIOEN Program establishes partnerships with industry for cooperative R&D activities with public laboratories at universities and research institutes co-funded by FAPESP and industry.

Research goals are specified in accordance with the interests of private partners and FAPESP's commitment to support high quality research in the State of São Paulo. Research agencies from federal and other state government agencies such as CNPq and FAPEMIG respectively, participate in the BIOEN Program and other agencies are expected to join.

The
FAPESP BIOENERGY PROGRAM BIOEN

comprises five Divisions:

BIOMASS RESEARCH

Focus on Sugarcane, including genomics, biochemistry, cell biology, physiology, plant breeding and sugarcane farming technologies

ETHANOL TECHNOLOGIES RESEARCH

Focus on Processing and Engineering

ALCOHOLCHEMISTRY AND BIOREFINERIES

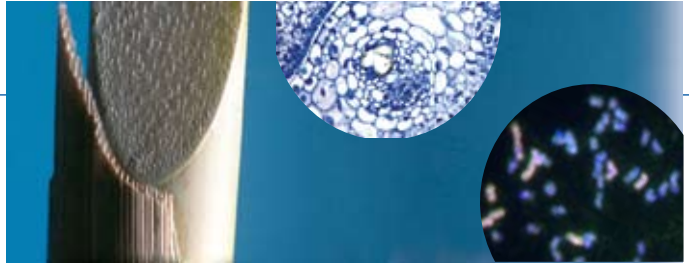
Integrated focus on sugarchemistry, alcoholchemistry and bio-products

ENGINES

Focus on ethanol applications for motor vehicles: Otto cycle engines and fuel cells

IMPACTS

Focus on social, economic and environmental studies, land use, intellectual property associated with the biofuel industry



BIOEN

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BIOMASS RESEARCH DIVISION

http://bioenfapesp.org

The goal of the Biomass Research Division is to develop basic research on sugarcane and other plant species suitable for producing biofuel, to establish an interface between different disciplines and to produce tools that will allow a systems biology approach. The research projects associated with biomass should address the main problems of plant biology, trying to elucidate the relationship between genome, metabolism, and the physiological and adaptive responses to the environment.

The purpose is to identify new pathways to genetically manipulate the energy metabolism of cultivated plants, creating new biofuel alternatives. Ultimately, sugarcane biology will be addressed taking into consideration aspects of genomics, photosynthesis, carbon storage and partitioning, existing genetic diversity and potential use of these discoveries in breeding programs. Transference of academic knowledge to the end-users is an important challenge that needs to be tackled in order to produce an energy cane.

The proposed activities also include research on organisms with lignocellulolytic activity with a potential use in cellulosic ethanol production. The challenge is to establish a new model of research and development in the area of bioenergy that will have an effective impact on the improvement of cultivars of interest.

MAIN OBJECTIVE

To identify new paths to genetically manipulate the energy metabolism of crops creating new biofuel alternatives.

RESEARCH AGENDA

- Uncover metabolic networks related to the production of carbohydrates and sucrose through the use of "omics" technologies.
- Integrate the results in a single platform and develop bioinformatic tools to assess the information.
- Discovery of genes associated with agronomic characteristics of interest.
- Development of new sugarcane cultivars.
- Signaling, regulation of gene expression and regulatory networks.
- Genetic transformation of sugarcane and other grasses.
- Molecular markers and breeding.
- Sequencing, physical, genetic and molecular mapping of genomes.
- Understand cell wall structure, architecture and biological function.
- Discover new cellulolytic fungi species capable of degrading biomass.
- Refine field practices for enhancing crop production including soil management, fertilization and precision agriculture.
- Improve control of weed, pests, and diseases through chemical or biological control, the development of resistant varieties and adequate field practices.

BIOEN

FAPESP

ETHANOL INDUSTRIAL TECHNOLOGIES RESEARCH DIVISION

http://bioenfapesp.org

Engineering, processing and equipment design related to bioethanol production including Cellulosic Ethanol

MAIN OBJECTIVE

Increase productivity (amount of ethanol per ton of sugarcane), energy saving, water saving and minimizing environmental impacts.

RESEARCH AGENDA

- Identify and improve bottlenecks in the ethanol production chain at the mill (reception, juice extraction, hydrolysis process, among others).
- Improve fermentation process yield.
- Optimization of water recycling and energy efficiency.
- Improve separation processes especially to dehydrated ethanol.
- Develop cellulosic ethanol technology focusing on the following objectives:
  - Characterization and development of physical-chemical pre-treatment of bagasse for ligninocellulose hydrolysis;
  - Development of acid catalyzed and biocatalyzed saccharification;
  - Development of high performance cellulases and hydrolases;
  - Reduction of the impact of fermentation inhibitors;
  - Development of microorganism strains capable of efficient fermentation of pentoses and hexoses; by-products recovery.

BIOEN

FAPESP

ALCOHOLCHEMISTRY AND BIOREFINERIES DIVISION

http://bioenfapesp.org

Develop further the concept of ethanol as a renewable source of energy. Design and develop industrial units near sugar and alcohol producing facilities that take advantage of renewable raw materials available (ethanol, sugar, CO_2, bagasse, trash, yeasts) to produce and design high added value products.

Sugarchemistry for intermediate chemical production.

Alcoholchemistry as a petrochemistry alternative.

MAIN OBJECTIVE

Substitute, as much as possible, fossil derived compounds

RESEARCH AGENDA

- Production of ethanol and biodiesel, by intercropping of oleaginous crops in areas of sugarcane plantation renovation.
- Develop products from ethanol via acetaldehyde and ethylene route.
- Undertake chemical synthesis of intermediate oxygenated chemicals (alcohols, acid ketones), polymers (PHA, lactic acid), nutraceuticals directly from sucrose.
- Develop biocatalysis for transformations of carbohydrates in valuable chemicals.

BIOEN

FAPESP

ENGINES DIVISION

http://bioenfapesp.org

Ethanol, as a renewable source of energy, has been used, almost exclusively, as a fuel for internal combustion engines to power vehicles. Reducing carbon emission is a global aim to the automotive industry. The introduction of flex-fuel vehicles in the automotive market immediately revitalized the use of ethanol as customers benefited from freedom of choice.

Strong competitors exist such as hybrid-electric vehicles powered by highly efficient diesel engines or direct injection gasoline engines, the application of second generation renewable fuels such as dimethyl ether or isobutanol and the use of electric vehicles powered by batteries or fuel cells.

MAIN OBJECTIVE

To develop new engine configurations that efficiently use renewable sources of energy focusing on combustion and fuel cells.

RESEARCH AGENDA

- Consolidate ethanol as the renewable substitute for gasoline in the short to medium term (10 to 20 years), with the evolution of internal combustion engines, and in the long term with fuel cells.
- Design flex-fuel engines with the same performance, consumption, pollutant emissions and durability as engines that run on a particular fuel blend.
- Solve the cold-starting problem associated with the use of pure ethanol.
- Develop ethanol or sugarcane products with physical-chemical properties adequate for compression ignition engines.

BIOEN

FAPESP

IMPACTS RESEARCH DIVISION

http://bioenfapesp.org

IMPACTS AND OUTREACH ACTIVITIES

HORIZONTAL THEMES: SOCIAL AND ECONOMIC IMPACTS, ENVIRONMENTAL STUDIES, AND LAND USE

To consolidate biofuel production one needs to understand the local and global market, patterns of bioenergy diffusion, institutional incentives, rules and industrial public policies. The expansion of ethanol production in Brazil, for instance, will combine productive gains based on new technologies and increase of sugarcane planted area. Fast rates of growth could generate negative impacts on environment, on social relations and other economic activities.

It is important to analyze risks and to propose specific methods or policies to minimize these impacts. Additionally, as far as ethanol becomes a global strategic fuel and a widespread option to help with issues related to the challenges of global climate change, topics such as carbon and energy balances and greenhouse gases emissions gain special relevance.

MAIN OBJECTIVE

- Risk assessment of ethanol as a renewable source of energy.
- Certification Methodology for ethanol produced in a sustainable environmentally friendly manner.
- Research into new agronomical practices (precision agriculture, mechanization, no-till farming, low input practices, new crop protection systems) and their impact on soil loss, management and efficiency in different production environments.
- Improve recycling of plant nutrients from crop and industry residues in the sugarcane farm and industry system.
- Define changes in carbon sequestration, greenhouse gases emission gains, carbon and energy balances impacted through the use of Bioenergy.
- Evaluate the environmental impact of GM sugarcane and biosafety.
- Risk assessment of effects on environment, on social relations and other economic activities (competition with food supply, energy supply and local materials).

PROJECTS

SUGARS AND GLYCOLS FROM HYDROLYSIS AND DELIGNIFICATION OF SUGARCANE BAGASSE AND STRAW AND RAPID CHARACTERIZATION OF LIGNOCELLULOSICS

Adilson Roberto GONÇALVES
Lorena School of Engineering / University of São Paulo (USP)

Figure 1. Reactor for hydrolysis and delignification

The Project has four main objectives:

1. Development of a rapid method for the characterization of lignocellulosics using analytical pyrolysis together with GC-MS and classical methods;

2. Pre-treatments studies for the hydrolysis of cellulose using both steam-treatment process for animal feed production and mild-acidic conditions hydrolysis;

3. Hydrogenolysis of sugars to polyols by means of hydride transfer reaction (formate reaction);

4. Lignin solvolysis by organosolv and soda processes.

MAIN PUBLICATIONS

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TOPOCHEMISTRY, POROSITY AND CHEMICAL COMPOSITION DETERMINING SUCCESSFUL ENZYMATIC SACCHARIFICATION OF SUGARCANE BAGASSE

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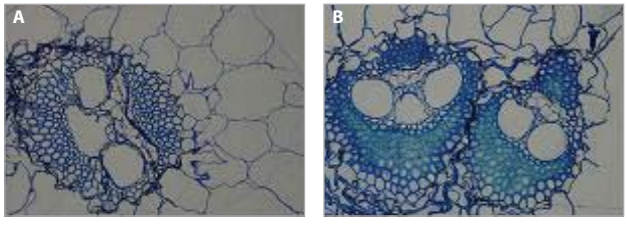

Figure 1. Complexity of sugarcane anatomy. (A) pith; (B) rind

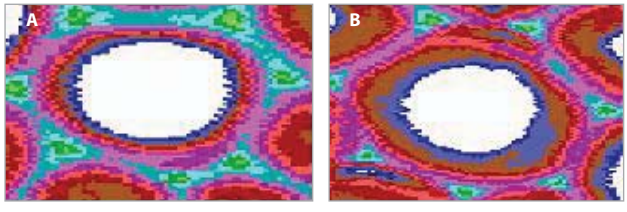

Figure 2. Topochemical distribution of lignin in rind fibers of sugarcane (A) and 1-h chlorite delignified sugarcane (B). In these pictures, blue and brown colors means low lignin contents, while red-pink and light green colors means high lignin contents

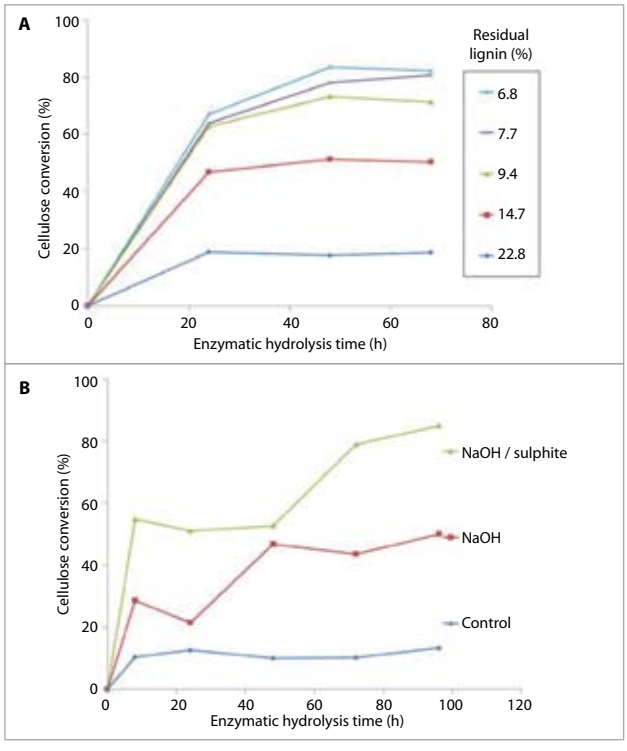

Figure 3. Effect of chlorite treatment time (A) and alkaline and alkaline/sulphite chemothermomechanical pretreatment (B) on time-dependent cellulose conversion of bagasse

This project proposes the development of a new concept for saccharification of sugarcane bagasse based on the enzymatic hydrolysis of forthcoming plants down regulated on lignin biosynthesis. The hypothesis is that once a sugarcane bagasse with reduced lignin content would be available, the hydrolysis of the entire polysaccharide fraction could be performed at mild conditions. Nowadays, the methods used in the pre-treatment of lignocellulosic materials, which precedes the advantageous enzymatic hydrolysis are quite harsh and inevitably lead to degradation and loss of valuable carbohydrates. Moreover, they are generally energy-intensive and generate undesirable by-products, which significantly add processing costs. To check on this hypothesis, the present proposal plan to prepare sugarcane bagasse samples with progressively reduced lignin contents by using a selective chemical step followed by mechanical fiberizing. These bagasse samples would serve as models to find the desirable characteristics, mainly in terms of lignin content, lignin topochemistry and cell wall porosity, necessary to minimize the harshness or abolish the treatment that precedes the enzymatic hydrolysis of the available polysaccharides, namely hemicellulose and cellulose. A subsequent step will be the evaluation of the plants with decreased lignin contents from studies involving down-regulation of lignin biosynthesis or from hybrids selected for low lignin contents.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Sugarcane cell anatomy is complex. Vessels are surrounded by fiber bundles. This cell groups are distributed along all internodes in the stalk. The inner part of the internodes named pith (*Figure 1A*) contains few of this cell groups and a large number of wide-thin-walled parenchyma cells. On the other hand, the outermost part, usually named rind, contains a large number of fiber bundles and smaller parenchyma cells (*Figure 1B*).

This morphological variation is relevant for all intents of sugarcane bagasse hydrolysis. Certainly, lignin and polysaccharide distribution varies in these different cell walls. Cellular UV-microspectrophotometry from untreated cell walls showed the presence of ferulic and p-coumaric acids linked to lignin or arabinoxylans. Vessels presented the most lignified cell walls followed by fibers and parenchyma. Pith parenchyma is not extensively lignified but contains significant amounts of ferulic and p-coumaric acids.

Cellular images showed highest lignin concentration in middle lamella and cell corners. One-hour chlorite treatment promoted rapid delignification and ferulic acid removal on parenchyma cell walls, while thicker fiber cell walls (*Figure 2A*) were only slightly delignified after 1-h treatment (*Figure 2B*).

Current breeding programs already provide sugarcane lines with lignin content as low as 16% compared to 25% in control plants. These plants are under study in the current project. However, first results have been obtained with chlorite delignified models and alkali-treated bagasse. Enzymatic hydrolysis of the entire sugarcane bagasse with 10 FPU Celluclast/g of dry material for 48 h resulted in the hydrolysis of 21.5% and 66.6% of the original cellulose in the untreated (22.8% lignin) and 2h-chlorite treated sugarcane bagasse (9.4% lignin), respectively (*Figure 3A*). Removal of 33% of lignin and 13% of hemicelluloses by NaOH pre-cooking improved saccharification levels to 50%. Alkaline-sulphite pre-cooking increased lignin and hemicellulose removal to 53% and 29%, respectively, reaching 85% saccharification after 96h of enzymatic hydrolysis (*Figure 3B*). Treated samples seem to simulate well the plants with reduced lignin content. In a subsequent step, sugarcane lines with reduced lignin content would be evaluated under similar hydrolysis conditions.

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YEAST IMPROVEMENT BY METABOLIC AND EVOLUTIONARY ENGINEERING

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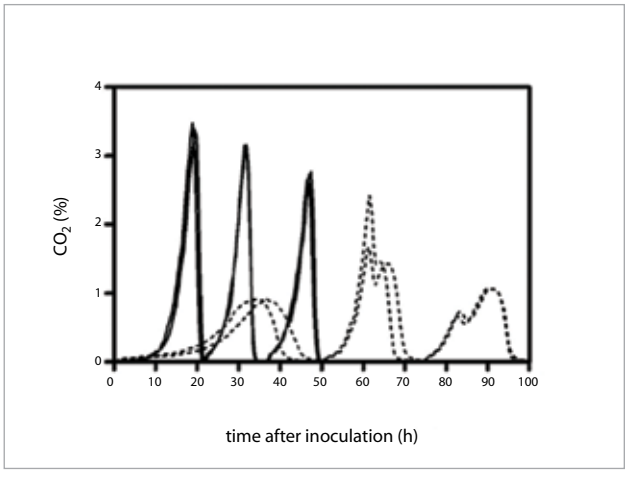

Figure 1. CO_2 profiles during evolution of Saccharomyces cerevisiae *in Sequential Batch Reactors*

Using evolutionary and metabolic engineering, either individually or in combination, our global aim is to improve yeast for its use in biorefineries. First-generation bioethanol production in Brazil, in which sucrose from sugarcane is converted into ethanol by *Saccharomyces cerevisiae* with high yields, was chosen as a first case-study. We started with a yeast strain which had already been metabolically engineered to hydrolyze sucrose exclusively in the intracellular environment (Prof. Boris Stambuk, Federal University of Santa Catarina, Brazil). Without the capacity of hydrolysing sucrose extracellularly, this strain is obliged to transport this sugar actively into the cells via symport, which causes ATP expenditure to extrude protons from the cells back to the culture medium, in order to avoid acidification of the citoplasm. This energy drain forces the cells to produce more ATP, which, under anaerobiosis, is basically coupled to ethanol formation. As a first aim, we will characterize this strain quantitatively, in order to demonstrate that it converts sucrose into ethanol with a higher yield, when compared to strains with normal invertase activity. Subsequently, this strain will be subjected to evolutionary engineering, in order to increase the ethanol yield on sucrose even further. Future studies will focus on the metabolic and evolutionary engineering of industrial yeast strains, with the aim of improving tolerance towards the most relavant stressors present in the industrial bioethanol production, such as high ethanol concentration, high temperature, high osmolarity, and acid environment. The improvement of second-generation biofuels will also be tackled, by investigating tolerance of yeast towards common inhibitors released during hydrolysis of lignocellulosic materials, such as acetate, furfural, and hydroxymethylfurfural.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The *Saccharomyces cerevisiae* strain that transports sucrose actively into the cells and hydrolyses it intracellularly was quantitatively characterized using a combination of chemostat cultivations and defined culture media. We were able to show that it delivers a 5% higher ethanol yield on sucrose, when compared to the reference strain (with normal extracellular invertase activity). Having achieved this first milestone, this strain is now being subjected to evolutionary engineering, both using chemostat cultivations and sequential batch reactors, aiming at improving the affinity of the active sucrose transport system. This, at least in theory, will increase the ethanol yield even further.

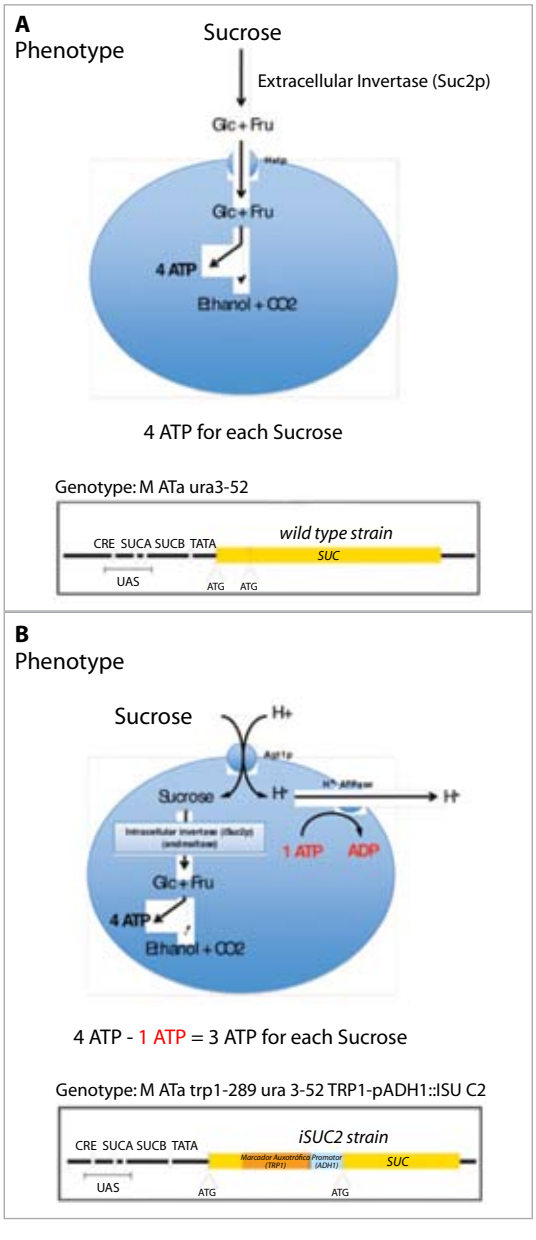

Figure 2. Sucrose uptake and metabolism, as well as the respective genotypes, in the reference strain (A) and in the modified strain (iSUC2) (B)

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SUGAR PRODUCTION BY ENZYMATIC HYDROLYSIS OF SUGARCANE BAGASSE

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Figure 1. Granulometric analysis of sugarcane bagasse

The modern chemical industry obtains more than 90% of the raw material for synthesis of organic molecules from oil. The rise in oil prices and the concerns and pressures of society to climatic changes, presenting challenges that this industry needs to overcome in the near future. The most viable alternative at this moment is the use of renewable raw material, such as lignocellulosic biomass available in the agricultural waste. The use of renewable resources represents an important development activity in emerging countries. However, these resources, natural and with high energy potential, are not properly exploited as a source of energy and raw materials for the chemical industry. The most important agricultural residues in the national territory, sugarcane bagasse certainly occupies a position of great prominence. Domestic production of sugarcane in 2009 reached about 569 million tons, destinated to the production of sugar and alcohol. It implies the recognition that the annual production of sugarcane bagasse reaches several huge numbers and the use of this waste is a national need. Therefore, the conversion of the cellulosic component of sugarcane bagasse into sugars, for them to be used as a source of raw material for the chemical industry is of great economic interest. In this sense, this project approaches the development of technology for the conversion of lignocellulosic fraction of sugarcane bagasse in a broth rich in sugars, which will serve as substrates for the production of specialty chemicals.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The intention of this project is to develop the technology for conversion of lignocellulosic residues (bagasse) in a product rich in fermentable sugars and a development that has a partnership with the Oxiteno Corporation and FAPESP.

The aim is to obtain a raw material for low cost, through biotechnology, adequate to produce various products with high added value of interest Oxiteno. Reducing the cost of raw materials is a key factor to boost the biotechnological production of chemicals (white biotechnology). The success of the project will attract investment in such projects for the country. Thus, not only the applicant company will increase its competitiveness in the market for specialty chemicals, but you can increase the competitiveness of the production of cane sugar.

The use of lignocellulosic biomass basically involves processes: pretreatment, hydrolysis of cellulose contained in the lignocellulosic materials into sugars and chemical or biotechnology transformation of these to obtain new products. In this development it was decided by the process of steam explosion as a technique of bagasse pretreatment.

The objective of this project is to study the production of sugars by enzymatic hydrolysis process of sugarcane bagasse pretreated with steam explosion. These sugars will serve as substrates for the production of specialty chemicals. To achieve the proposed objective will be developed the following steps:

- study of steam explosion pre-treatment;
- study the effect of bagasse delignification;
- study the process variables on the enzymatic hydrolysis kinetics of sugarcane bagasse.

MAIN PUBLICATIONS

The project is in its early stage of development and has been completed the steps of design, acquisition, installation and preliminary tests of the equipment for steam explosion of bagasse.

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SIMULATING LAND USE AND AGRICULTURE EXPANSION IN BRAZIL: FOOD, ENERGY, AGRO-INDUSTRIAL AND ENVIRONMENTAL IMPACTS

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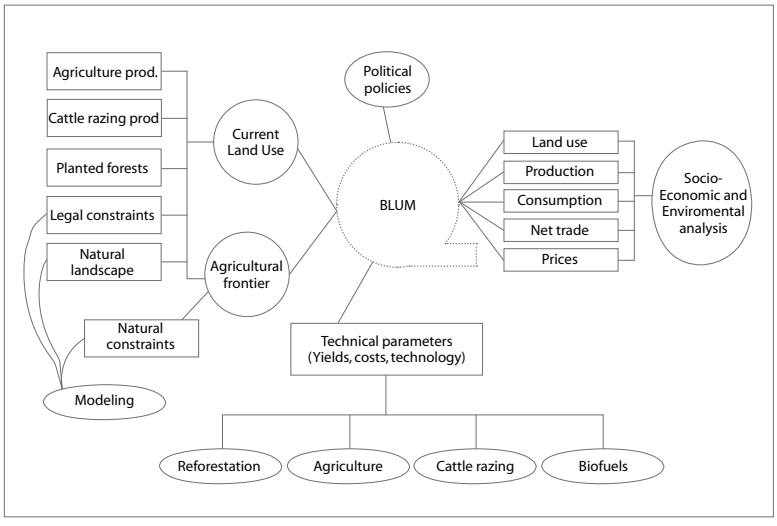
Brazilian agro-industrial output has experienced a cycle of strong expansion. The awareness that the era of cheap oil has reached an end and the growing energy concerns made the search for viable alternatives to hydrocarbon-based fuels into a global priority. The role of biofuels and bioenergy has shifted from a solution for reducing Green House Gases (GHG) emissions to the cause of rising food prices, environmental degradation and even threatening global food security.

At the same time, increasing income in developing countries has brought millions of people to increase their levels of food consumption, boosting international demand. National demand for agricultural products has also increased, pushed by biofuels (mainly ethanol) and grains for food and feed production. Within this context, Brazilian agricultural industry has responded to the world's need for food, feed, fiber and biofuels by both improving its yields and expanding cultivated area and investments.

To analyze this issues properly, in partnership with FAPRI (Food and Agricultural Policy Research Institute), ICONE developed an economic model called Brazilian Land Use Model (BLUM). The present application proposes the use and development of the BLUM for the food versus fuel debate. The BLUM will be sufficiently general to forecast all the main agricultural products in the entire national territory and, at the same time, detailed enough to deal with the (very) different regional characteristics of national territory.



Methodological diagram of Brazilian Land Use Model

With this project, ICONE wants to answer to the following questions: what will be the growth in sugar-cane planted area to respond to a growing demand? How will it affect land use change? What will be the main positive and negative environmental impacts? Is there any policy to be implemented by the government or the private sector to improve the rational use of the land?

The research on Indirect Land Use Change (ILUC) methodology is under continuous development. The BLUM has been improved to generate results on land use change instead of only land allocation. However, the need for additional improvements has been identified, which is the aim of this project.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

ICONE has been working in the BLUM since 2008 and have reached many advances and has established an intelligence network with specialists of several university research institutes in Brazil and abroad. The national network includes the Censoring Remote Center, at Federal Universiy of Minas Gerais (UFMG), various centers of Brazilian Agricultural Research Corporation (EMBRAPA), National Institute for Space Research (INPE), Center for Alternative Energy of Fortaleza (Centro de Energias Alternativas de Fortaleza), Sugarcane Technology Center (Centro de Tecnologia Canavieira – CTC), Luiz de Queiroz Agriculture School (ESALQ/USP), Laboratory of Remote Sensing (Laboratório de Sensoriamente Remoto) of Federal University of Goiás (LAPIG), among others. At the international level, we can mention the Center for Agricultural and Rural Development like the main partner and the World Bank (WB).

Main improvements developed so far in the model:

- ICONE innovated methodologically in, at least, four aspects. First, using satellite images (using GIS – Geographic Information System), which contributes to the incorporation of data of potential area for expansion of agribusiness, considering physical, environmental and legal restrictions – AgLUE-BR model (Esalq-USP). Second, projecting endogenously pasture area, which has not been considered in other land use models. Third, ICONE separates winter crops, which are planted after a primary crop in the same season. Finally, BLUM treats Brazilian agriculture dynamic considering six different regions and its peculiarities, which is essential for more accurate land use change analysis.
- Modification of the structure of the BLUM in order to respect the economic conditions of homogeneity, symmetry and adding up.
- BLUM was integrated to the international model and included into FAPRI's Outlook 2010.

Improvements to be developed in FAPESP project:

- Use deforestation data in the *Cerrado* Biome and land use, in order to estimate more trustworthy parameters (agricultural expansion over vegetation and substitution among different agricultural activities in the *Cerrado* Biome), based on empirical data.
- Estimate parameters using secondary data and satellite images for the different regions in the model.

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GENOMIC-ASSISTED BREEDING OF SUGARCANE: USING MOLECULAR MARKERS FOR UNDERSTANDING THE GENETIC ARCHITECTURE OF QUANTITATIVE TRAITS AND IMPLEMENT MARKER ASSISTED SELECTION

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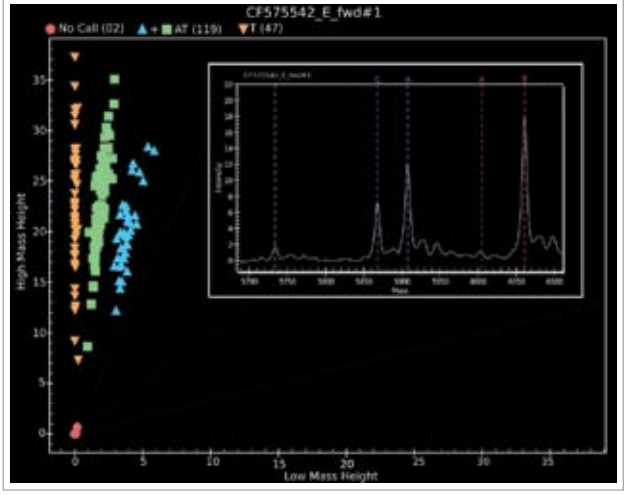

Figure 1. Example of results obtained with the SNP technology. Upper right: results for a single individual, showing the results for two loci; each peak corresponds to a nucleotide (A and C for the first, A and T for the second, that is homozygous). Left: genotyping of the segregating population, showing 3 classes

Breeding programs have been successfully over the years in the generation of new improved sugarcane varieties (*Saccharum spp.*), which are more productive and resistant to pests, diseases and abiotic stresses. These varieties are of central importance for sugar and ethanol production. However, breeding process takes about 10 to 15 years to release new varieties, mainly because of the difficulty to correctly identify good genotypes on the fields, since there is strong influence of environmental conditions. This process could be speed up with the development and the use of genetic markers, which are genomic regions that could be observed (evaluated) on each individual. By studying the segregation of those markers, it is possible to estimate the genetic distances between them, resulting in the so called genetic maps. After, linkage studies are performed in order to associate genotype (based on molecular traits) and phenotypes (traits that are evaluated on field conditions, such as sugar and fiber content). If the genomic regions are strongly linked with genes that control agronomic traits, they could be used for help the breeding process. Since most of the traits of agronomic and economic importance are quantitative (controlled by many loci), the major goal is to identify genomic regions associated with such traits, named quantitative trait loci (QTL). The use of markers in genetic studies, including QTL mapping, has allowed important progress in the knowledge of the genomic structure, genetics and evolution of sugarcane. In this project, new markers will be developed and used for QTL mapping. A new class of very useful markers called genic molecular markers (GMMs) will be developed and used. This kind of marker (EST-SSRs and SNPs), also named Functional Markers, will be obtained from expressed sequences from the SUCEST data bank, and sequencing genes from BAC clones. BAC libraries will be constructed using DNA from parental sugarcane varieties employed in the biparental crosses used for genetic mapping. The GMMs developed will be used for: 1) QTL mapping in biparental crosses; 2) association studies using sugarcane genotypes important in breeding programs, using a panel of about 150 genotypes important for Brazilian breeding programs. Once the genomic regions are found, strategies for marker assisted selection will be developed.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

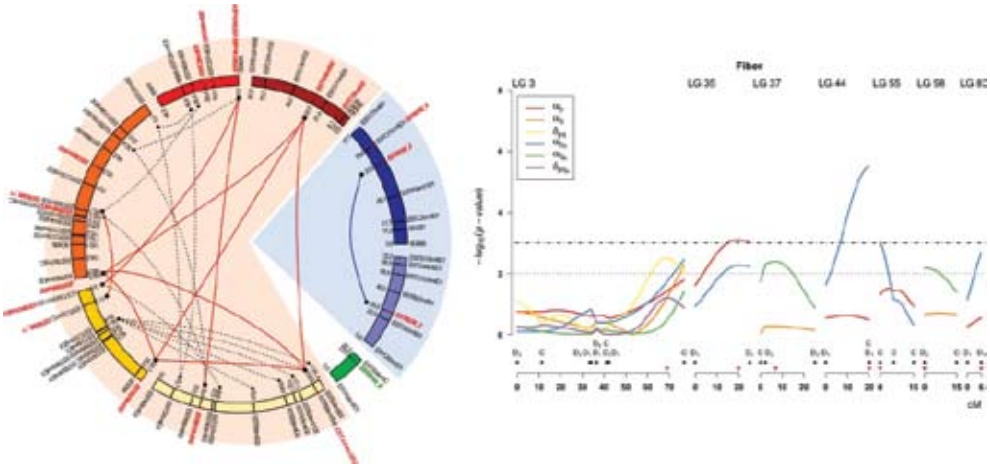

Figure 2. Example of mapping results. Left: Eight linkage groups obtained from markers with single (names in black), double (red) and triple dose (green). Lines are connecting the homology groups based on properties of the markers (EST-SSR and EST-RFLP) (dotted lines) or on loci with more than one copy on the genome (red and blue lines). Rigtht: QTL mapping for fiber content. Each curve indicates the statistical evidence of QTL presence. Genetic effects for each parent, location and harvest were investigated

DNA from 220 individuals from the mapping population was extracted and quantified. The same was done with the genotypes from the association mapping panel. About 350 SNPs developed by international collaborators (Southern Cross University, Australia) were used to genotype the parents of the mapping population, as well as 14 random selected individuals from the progeny. This was done to check if the SNPs were polymorphic, i. e., if they were segregating on the population, providing information for the mapping studies. About one third of the SNPs are informative, a number which should be considered satisfactory, given that the SNPs were developed for Australian varieties. Also, Dr. Gláucia Souza provided information about 438 EST sequences that showed differential gene expression, and from them about 2200 SNPs were found, showing that this marker has a great potential for sugarcane studies, due to its abundance. At this time, more sequences have been evaluated with the goal of having at least 700 polymorphic SNPs.

So far, about 40 SNPs were used to genotype the whole biparental mapping population. Several challenges arose, due to the genome complexity of sugarcane, that is a polyploid species. First, the software that is provided with the SNP technology that has been used (Sequenom Inc.) was developed for diploids, and can not be directly used to interpret sugarcane data. Therefore, alternatives were investigated and a new computer program is under development. Second, the SNP technology allows the usage of markers with higher doses, i. e., with more than one copy on the polyploid genome. Statistical methods available to analyze this type of data are not satisfactory, since they are based on unrealistic biological and statistical assumptions. Several new methods were then developed to estimate the dose of the markers, to allocate them in the genetic maps and to estimate homology groups. Third, currently none of the models used for QTL mapping can correctly deal with information from these new maps with a mixture of several doses. The common approach is based on single marker analysis of only simplex loci. Clearly this is not adequate for modern data and do not allow studies of the genetic architecture of quantitative traits in sugarcane. Several alternatives have been investigated, including multiple interval mapping and mixed models. The results are promising and will likely give a significant contribution in a near future.

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DEVELOPMENT OF ANALYTICAL METHODOLOGIES AND ORGANOSOLV DELIGNIFICATION PROCESSES APPLIED TO BAGASSE AND STRAW FROM SUGARCANE

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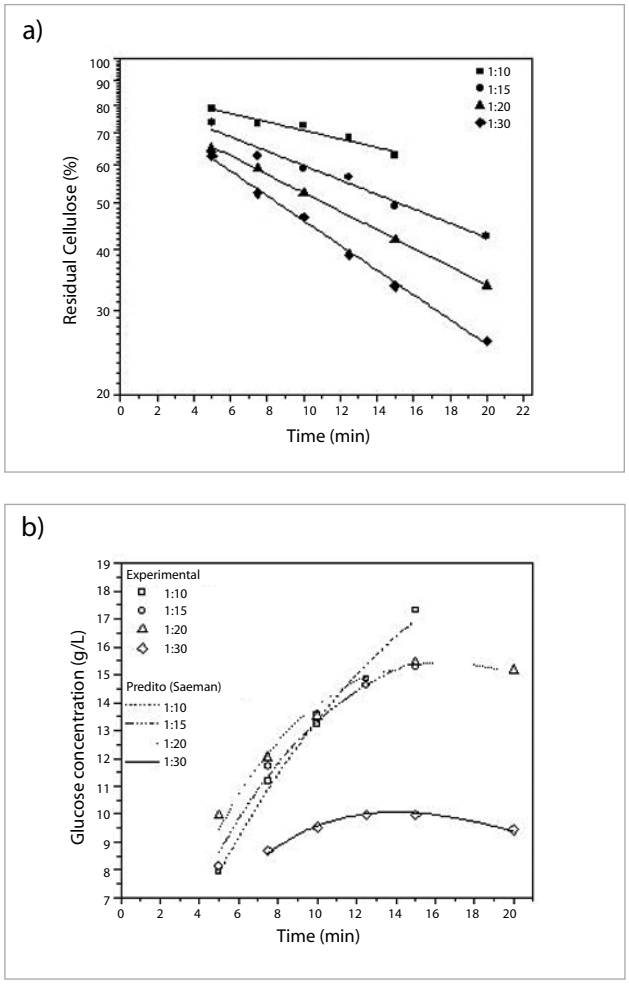

Figure 1. Hydrolysis of sugarcane bagasse pulp (fiber fraction) at 215ºC with H_2SO_4 0.07% and different solid to liquid ratios. (a) Semi logarithm plot of weight loss versus *reaction time and (b) Glucose concentration at the same reaction times*

The proposals in this project were formulated based on scientific output and experience accumulated over 20 years dedicated to the study of characterization and production of cellulose and lignin derivatives from lignocellulosic materials, with special attention to the bagasse obtained from sugarcane processing. The proposed project is focused on two main aspects: the study of lignin's solvency through delignification reactions and the development of analytical methodology for the characterization of the main components of lignocellulosic materials, particularly applied to bagasse and straw from cane sugar. The delignification processes will be investigated as pre-treatment step for polysaccharide's hydrolysis with special attention to the production of sugars from cellulose and also to recovery of lignin present in sugarcane bagasse for further industrial/commercial utilization.

Lignin's solvency will be studied using three basic processes: conventional (aqueous solutions), organosolv and organosolv assisted by supercritical fluids. As a result of these procedures, the cellulosic pulps will be employed for the production of glucose by means of acid hydrolysis of cellulose. The technique that uses fluids in the supercritical state may allow the hydrolysis of sugar without the prior separation of the lignin present in the raw materials (straw and bagasse from sugarcane).

The traditional methods for the characterization of lignocellulosic materials were developed and optimized for wood and wood derivatives (pulp and paper). Due to the different characteristics of grasses and agricultural wastes (especially from crushed cane sugar industry) the modifications of existing methods and/or development of new analytical methodologies for the chemical characterization of sugarcane bagasse is of fundamental importance for both academic studies and industrial applications.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The lack of specific methodology for the characterization of sugarcane bagasse and straw leads to inadequate results and hamper both the planning of industrial applications and the interpretation of analytical data. Thus, the main aim of this study was to develop specific analytical methodologies to the chemical characterization of sugarcane bagasse. The determination of lignin was studied by the hydrolysis and dissolution of the polysaccharide fraction in sulfuric acid solutions. The sugars and derivatives of these hydrolysates were analyzed by high performance liquid chromatography (HPLC). Preliminary results showed different behavior between the sugarcane bagasse and its different fractions (fiber and pith cells) when submitted to acid treatments. The results showed the dependence of sulfuric acid concentration on lignin content determinations and the role of condensation reactions in the lignin characteristics. The gravimetric determination of lignin by Klason methodology (sulfuric acid solutions) showed that the optimum concentration of acid for determination of total insoluble lignin is in the range of 65 - 72% for all samples. Despite the similarities in chemical composition, klason lignins obtained from straw exhibited very low molar masses. Preliminary results obtained from holocellulose determinations showed also the need for optimized oxidation procedures in order to be successful applied to sugarcane bagasse analysis.

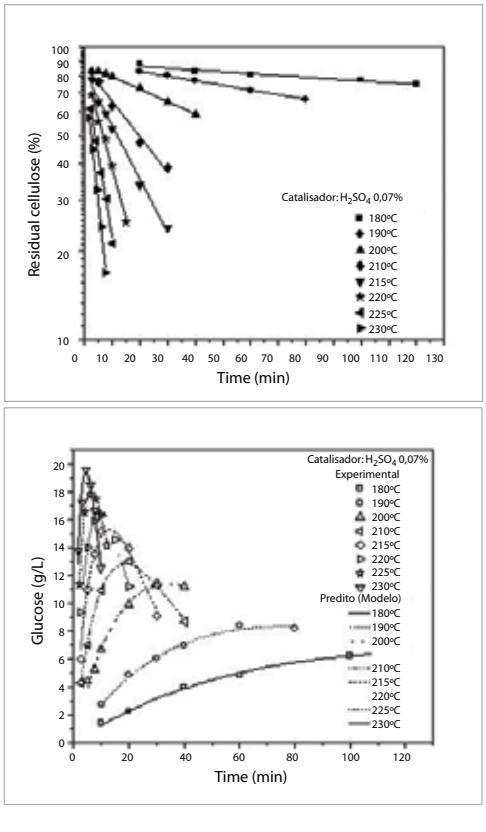

Figure 2. Hydrolysis of sugarcane bagasse pulp (fiber fraction) with H_2SO_4 0.07% at different temperatures (solid/liquid ratio = 1/10). (a) Semi logarithm plot of weight loss versus reaction time and (b) Glucose concentration at the same reaction times

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PHASE EQUILIBRIUM AND PURIFICATION PROCESSES IN THE PRODUCTION OF BIOFUELS AND BIOCOMPOUNDS

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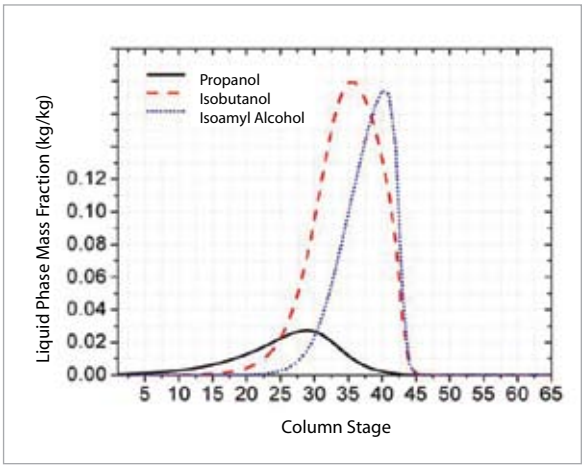

Figure 1. Concentration profiles of high alcohols in bioethanol distillation columns BB1 (Batista and Meirelles, 2009)

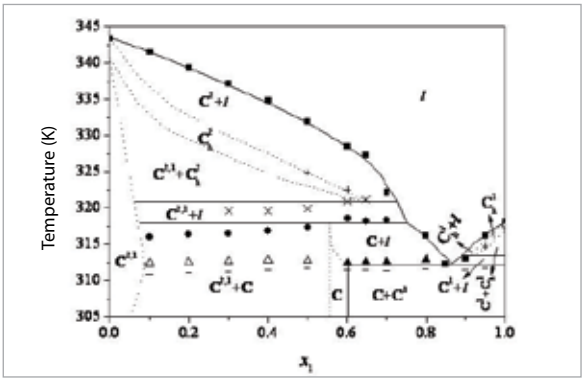

Figure 2. Phase diagram for the system lauric acid (1) + stearic acid (2). (■) fusion temperature; (●) peritectic temperature; (▲) eutectic temperature; (×) metatectic temperature; (+), (△), (–) transitions temperatures on the solid phase; (○) transition on the solid phase of the pure component (Costa et al., 2009)

This project aims to optimize the purification processes used in the production of biofuels and to enhance the added value of byproducts generated during such processes. In the case of bioethanol, the distillation process will be investigated taking into account minor components relevant for product quality, according to standards fixed by the legislation for biofuels and the requirements of the raw materials for the chemical, pharmaceutical, cosmetics and food industries. The configurations of distillation columns currently in use in sugar mills will be investigated by experimental means in the industrial units and by process simulation. A comprehensive investigation of the phase equilibrium of wine will be carried out, taking into account a complete set of minor components. New and innovative configurations for distillation columns will be proposed, aiming at better product quality, equipment flexibility, higher ethanol recovery, lower energy consumption and better byproduct quality. Such configurations will be further tested for concentrating the bioethanol obtained from wine with a high alcohol content and from cellulosic residues. In the case of ethylic biodiesel, a comprehensive investigation of the different types of phase equilibrium occurring throughout the whole production process will be carried out. The use of ethylic alcohol as a solvent for extracting vegetable oils from seeds and grains, and for deacidifying crude oils by liquid-liquid extraction, as well as its use as a reactant in biodiesel production will be studied, with the purpose of integrating biodiesel and bioethanol productions. The optimization of the whole production process, including the oil extraction and deacidification, biodiesel reaction and purification, will be performed by simulation. In the case of biocompounds, strategies for enhancing the value of byproducts generated during the production of biofuels will be investigated. For instance, the fractionation of higher alcohols generated as a sidestream during the distillation of bioethanol, the use of glycerol in the production of surfactants and emulsifiers, the recovery of nutraceuticals from edible oils, and the formulation and fractionation of fatty mixtures based on solid-liquid equilibrium data.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The binary distillation of bioethanol is a frequent research topic found in the literature. However its distillation taking into account the real complexities of wine composition and the industrial column configurations is a subject largely unexplored. The simulation tools available nowadays make it possible to reproduce the industrial process with a high degree of reliability, providing a firm basis for optimizing it and suggesting new configurations that can improve the efficiency of the bioethanol distillation. The distillation behavior of several minor components, classified into light, middle volatility and heavy compounds, were investigated in the production of spirits, hydrated ethanol and neutral alcohol. Middle volatility components, despite their very low content in the original wine, achieve high concentrations in specific parts of the distillation column (*Figure 1*), affecting the whole concentration process in a significant way. Strategies for controlling bioethanol contamination with light components were also developed.

Due to several drawbacks, ethylic biodiesel is almost not produced on an industrial scale. If these drawbacks were solved, an approach based on the use of bioethanol in several steps of biodiesel production, from seed to tank, would become technically feasible. Traditional and innovative techniques for deacidifying crude vegetable oils were investigated by experimental trials and simulation. The innovative techniques used bioethanol as a solvent or extractant. The biodiesel reaction occurs in a two-phase environment, requiring information on the corresponding equilibrium data. Such data were measured and correlated in situations suitable for homogeneous catalysis and biocatalysis.

The production of biofuels generates byproducts whose added value can be increased by fractionation or transformation. Phase equilibrium data provide the basis for optimizing the purification process and product formulation. The physical-chemical properties and equilibrium data were correlated and measured for fatty mixtures containing fatty acids, fatty esters, fatty alcohols, triacylglycerols and nutraceutical compounds (*Figure 2*).

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CONTRIBUTION TO THE PERFORMANCE IMPROVEMENT OF THE INDUSTRIAL PROCESS FOR OBTAINING ETHANOL FROM SUGARCANE BY USING MICROWAVE AND ULTRASONIC ENERGIES

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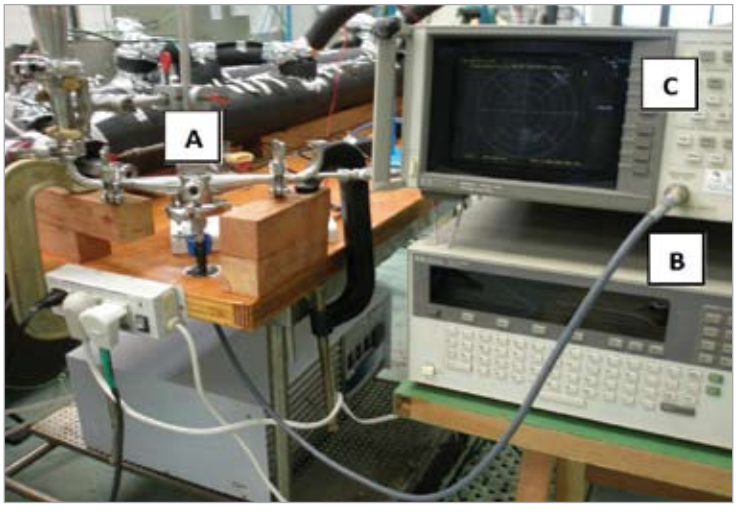

Figure1. Assembly for continuously testing dielectric properties of the sugarcane must as functions of temperature: (A) probe cell; (B) connecting cable; (C) network analyzer

The present study consists of searching techniques to improve the productive capacity of the ethanol industry, by developing new technologies based on the application of microwaves and ultrasound, envisaging a better performance during the fermentative process. In order to achieve that target, one of the main objectives is to pasteurize the sugarcane must before fermentation. The must is composed by the mixture of the sugarcane juice and the syrup coming from milling the cane and from the sugar manufacturing, respectively. Such a must carries a heavy microbiological load made up of bacteria and wild yeasts. The presence of bacteria into the fermentation vats is associated to the decreasing of the fermentation performance, because part of the substrate is wasted to make others products like acetic and lactic acids, thus decaying the quality of the ethanol. Besides, bacteria may induce the occurrence of ferment flocculation, bringing a series of drawbacks to the process, such as yield reduction, expenditures with additives and bactericides, decrease of productivity, among others. On the other hand, wild yeasts are mostly flocculants by its nature, exhibiting low ethanol productivity and high multiplication rate. The industrial ferments utilized in most of the Brazilian sugar mills, selected in conformity to their excellent fermentation potential at the beginning of the harvest season, are rapidly substituted by the wild yeasts, changing the process performance. Pasteurizing by microwaves is an efficient and rapid method, easily adaptable to the present ethanol plants, where the majority of equipment operations are based on batch processes. In order to aggregate efficiency to the fermentation process after microwave pre-pasteurization, it is suggested applying ultrasonic energy: a few research works have already shown that its ministration under controlled conditions can accelerate the metabolism of *Saccharomyces cerevisiae*, among other capabilities, although this kind of energy for stimulating fermentation has not found any industrial scale application yet.

The combination of the two technologies could be offering significant contribution to improve the ethanol production. Besides developing new technologies for the sugar-alcohol industrial sector, employing microwave and sonic energies signifies to take advantage of clean energies that can be obtained by co-generation from the surplus energetic sources of the sugarcane mills.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

1. Dielectric properties

The interaction between electromagnetic energy and the constituents of a dielectric material converts microwave energy into thermal energy, by means of several mechanisms of molecular and atomic scale. In order to determine the dielectric parameters of the sugarcane must, which also varies with temperature and frequency of the electrical field, a special cell was designed and developed so as to lodge the probe of a system for measuring dielectric properties, allowing the fluid to circulate continuously through it, inserted into a suitable circuit (*Figure 1*). Flow-rates and temperatures can be adjusted during the operation of this particular system, for continuously determining its dielectric properties within the range of microwave frequencies from 300 kHz to 6 GHz, as functions of temperature.

2. Lethality parameters

The lethality parameters, known as D and z values, specify times and temperatures needed to destroy the deteriorating target microorganisms, making possible to determine equivalent values of pasteurization at any other temperature.

Experiments are being run either in a bench top microwave reactor (CEM Discover) or in a Heated Circulating Bath. Preliminary tests were conducted by using D = 0,5 min and z = 4,5ºC for Saccharomyces cerevisiae as a reference (Stumbo, 1973)*. Glass tubes with distilled water were heated in the microwave reactor, with agitation. At the desired temperatures, a solution of industrial yeast with initial controlled count was added to the tubes and exposed to microwaves to evaluate the cells reduction. The counting of cells and cellular viability (method of coloring) were done with a microscope and Neubauer chamber for confirming the reduction of the initial count. The values adopted for D and z were shown to be adequate, with experiments and entire development of methodology being presently in course.

* STUMBO, C.R. Thermobacteriology in Food Processing, 2nd Ed, Academic Press, New York, 1973.

3. Expected Results

This study proposes to improving the fermentation process of sugarcane must for obtaining ethanol by means of: time and waste reduction in the process; processing of a better quality product, characterized by less contaminants of ethanol; decreasing of acid treatment for ferment deflocculation; reducing the employment of bactericide agents; clean energy utilization, possible of being obtained by co-generation through taking advantage of the bagasse from sugarcane.

It is expected through this study to get significant improvements in obtaining products and processes for the bio-combustibles fabrication, specifically ethanol, keeping the Brazilian leadership in the production of bio-ethanol.

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GENE EXPRESSION PROFILE AND CARBON ISOTOPE DISCRIMINATION IN SUGARCANE GENOTYPES UNDER WATER DEFICIT STRESS

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Figure 1. Overview of a field assay to evaluate cultivars under water deficit conditions of. Photograph kindly provided by the Centro de Cana (IAC), Ribeirão Preto, SP

Sugarcane (*Saccharum spp.*) is major crop in Brazil as feedstock for the sugar and ethanol industries. To attend the increasing ethanol demand from external and internal markets, the sugarcane industry must expand the cultivated area, incorporating land from 'cerrado' and pastures from Southeast and Western Central Brazil, characterized by a dry winter with a prolonged water deficit period. For the last 10 years, more than 80 sugarcane cultivars have been released in Brazil, but few with yield potential to be cultivated in drought-prone environments. Mechanisms of response and tolerance to water stress have been investigated in model plant species, whose genes were classified into two groups: one includes proteins that act directly on dehydration tolerance, and the other comprises regulatory genes. Previous work on sugarcane response to water deficit stress detected similar induced regulatory genes to the ones from rice and Arabidopsis, but structural genes associated with stress response have not been evaluated. Elucidation of sugarcane mechanisms involved in tolerance to water deficit would be valuable to develop cultivars productive and adapted to drought-prone regions, which could potentially assist in the sustainability of the sugarcane industry in these marginal regions. This proposal intends to establish an efficient and dependable method to evaluate water deficit stress in sugarcane by evaluation of several protocols, to enable the analysis of gene expression profiles between genotypes tolerant or susceptible to water stress using microarrays, followed by validation of differential gene expression by quantitative amplification of reversed transcripts (RT-qPCR). Analyses of marker gene expression (drought- or ABA-related structural or regulatory genes) will be conducted using RT-qPCR to validate the observed physiological responses. At the same time, ^{13}C discrimination technique (Δ) will be tested and optimized to evaluate the genetic diversity available for the trait, together with biochemical and physiological measurements, associated with water use efficiency and, consequently, water stress tolerance.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Our work has focused on three cultivars selected by the Centro de Cana from the Agronomic Institute of Campinas (IAC) under drought-prone environments, with one clearly more sensitive to drought, while the other two present contrasting behavior to water deficit in terms of biomass yield reduction. These cultivars have been used in various assays to standardize methods to evaluate physiological differences and gene expression associated with imposition of water stress deficit. The procedures evaluated so far included suspension of irrigation; addition of Polyethylene Glycol (PEG) to *in vitro* culture media; and indirect assay with methyl viologen (Paraquat).

The behavior of the three selected cultivars in response to irrigation withdraw under greenhouse conditions confirmed the expected performance obtained from field trials. The susceptible cultivar did not tolerate the 21-day period of exposure to water deficit, while the tolerant cultivar maintained and recovered growth. A similar difference between the tolerant cultivars was observed for the indirect evaluation based on Paraquat treatment. This *in vitro* assay using methyl viologen has been tested as a quick and indirect method to discriminate among genotypes for water deficit tolerance. Difference in tolerance to methyl viologen treatment is evaluated by placing leaf disks on a buffer solution containing this chemical (*Figure 2*), and the conductivity of the solution is evaluated. Differences among genotypes may derive from differences in tolerate oxidative stress. Differences in buffer solution conductivity was observed between cultivars, with large leakage of electrolytes from the sensitive cultivar under treatment with methyl viologen, while the less difference in conductivity was observed for the solution containing leaf disks from the tolerant cultivars. Similar assay has been conducted comparing an old *Saccharum officinarum* cultivar (Muntok Java), supposedly a more drought sensitive genotypes to a wild relative *Saccharum spontaneum* (SES), recognized as more tolerant to water deficit. There was little difference in buffer solution conductivity between both accessions suggesting that this assay might evaluate for only certain mechanisms which could be associated with drought tolerance.

The addition of PEG to culture media *in vitro* or *in vivo* has been adopted as a way to standardize stressful conditions by increasing osmotic potential. An assay to establish a suitable concentration of PEG 8000 to impose water stress indicated that 15% PEG was sufficient in a short period without harming the plants. Plants derived from this assay are being used to investigate the expression pattern of structural genes associated with stress response.

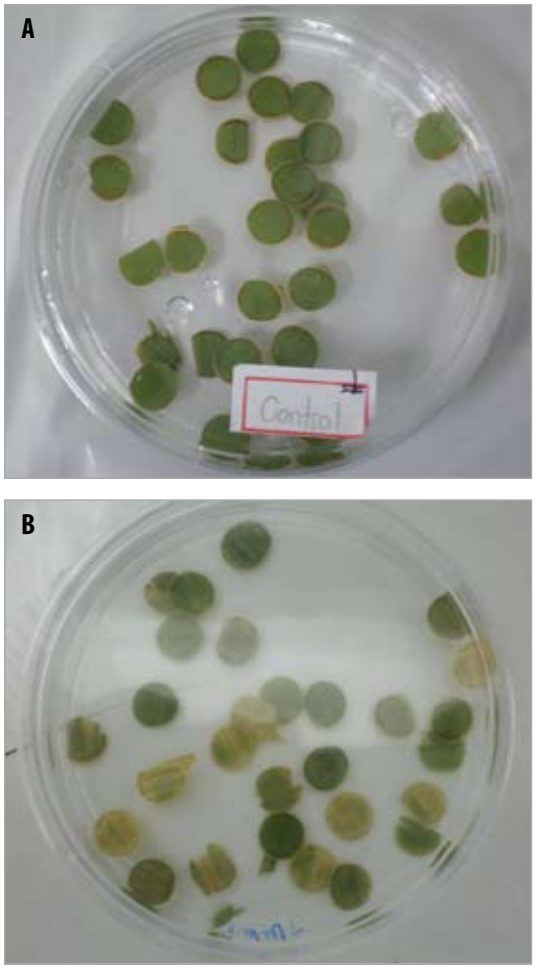

Figure 2. In vitro *assay to establish sensitivity to methyl viologen evaluated by electrolytic conductivity to be correlated with tolerance to water deficit. A) Leaf disks without treatment; B) same cultivar under 3 mM methyl viologen*

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ETHANOL PRODUCTION FROM SUGARCANE BAGASSE: ENZYMATIC HYDROLYSIS, MICROBIOLOGICAL ASSAYS TO EVALUATE TOLERANCE OF YEASTS TO THE TOXICITY OF HYDROLYSATES AND FERMENTATION AT HIGH TEMPERATURES

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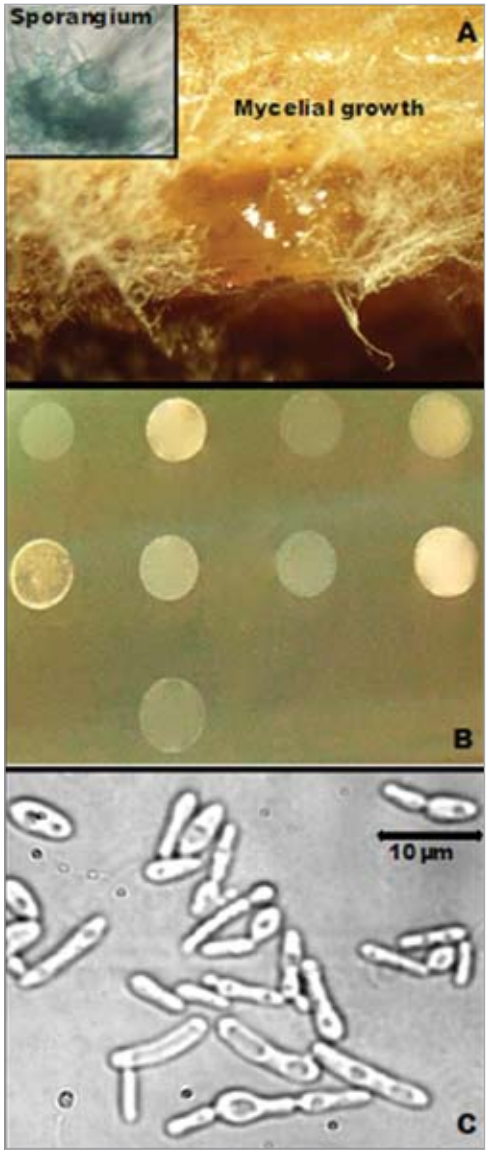

Figure 1. (A) a sporangium (phase contrast microscopy, 1000Í) and the mycelia growth (phase-contrast stereoscopy, 35Í) resulting from the propagation of Aspergillus nidulans *on sugarcane bagasse; (B) Selection of yeasts strains based on growth on plates containing high concentration of both sugar and acetic acid; (C) cells of* I. orientalis *able to convert glucose into ethanol in simple batch cultures at 42°C*

The hydrolysis of cellulolytic materials with diluted acids is well known, but this process generates toxic products of hydrolysis. Other negatives factors related to the acid hydrolysis are the corrosion and the high amounts of salts resulting from the acid neutralization. The production of enzymatic preparations at lower cost showing activity at lower pHs and resistance to its reuse are needed. In addition, the fermentation of cellulolytic hydrolysates depends on the yeast strain and the levels of toxic compounds present in the hydrolysates.

Physicochemical methods reported in literature for the pre-treatment and hydrolysis of bagasse as well as the use of crude preparations of cellulolytic enzymes produced by fungi will be evaluated and improved. The identification and quantification of the activity of each enzyme of the enzymatic complex able to hydrolyze the sugar-cane bagasse will be another target of this investigation. The production of ethanol by simultaneous saccharification and fermentation (SSF) of sugar-cane bagasse will be also studied.

There is a great need for the development of fast and reliable microbiological methods to assay yeasts strains and levels of the toxicity of the hydrolysates. Frequently, the circumstances preceding the arrest of the fermentation and types of changes of the fermentation profiles can provide valuable information. Assays have to be developed to predict how the fermentation will proceed. A synthetic medium will be optimized and used as a reference medium to study the effects of inhibitors produced during the bagasse hydrolysis and their interactions with respect to growth and fermentation using statistical methods. This medium will be used as a tool in fast diagnostic assays to evaluate the toxicity of the hydrolysates and the tolerance of the yeast strains to acidity and levels of inhibitors prior to the fermentation process. Solid media will be developed for the qualitative evaluation the toxic inhibitors of hydrolysis on the yeast growth capacity.

As temperatures greater than 30°C-34°C are observed in industrial reactors operating in tropical countries, the search for yeasts strains tolerant to acidity and high temperatures are required for hydrolysates fermentation. Strains tolerant to acidity and temperature will be used in the present study. Temperature usually aggravates the effects of other stress determinant factors. Assays in bioreactors will allow the optimization of the entire process for maximal efficiency of the ethanol production.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Among several fungi studied, *Aspergillus nidulans* was seen in the present work as the most promising fungi for the production of cellulolytic enzymes when grown on sugarcane bagasse pretreated with diluted acid at room temperature. The production of the CMCase was high in the culture inoculated with mycelia, while the avicelase showed greater activity in the culture containing spores. However, the same total cellulose activity was obtained from both mycelia and the spores cultures. The results obtained in the present work indicated that the filtrate from the *A. nidulans* cultures could be used as a crude cellulolytic preparation for saccharification of sugarcane bagasse and also as a medium for the ethanol production for containing high levels of total reducing sugar and low amounts of phenol.

Solid and liquid media were modified to study the effects of sugarcane bagasse inhibitors on yeast growth and fermentation. Some yeast strains were able to growth on plates containing high concentration of sugar and acetic acid, while growth of other strains was inhibited. Using a synthetic medium containing 18% glucose (w/v), the effects of increasing concentrations of acetic acid were evaluated. At this high sugar concentration, the growth decreased when the levels of the acetic acid increased up to 58 mM . Above this concentration, a low but constant biomass was obtained up to 330mM acetic acid added to the reference medium. The effects of the formic acid and furfural on ethanol secretion and growth were much greater than those obtained with acetic acid, while levulinic acid showed a lesser effect on growth and fermentation.

Issatchenkia orientalis is a non-Saccahromyces yeast able to convert simple sugars into ethanol at high temperatures and low pH values. Strains of this yeast were isolated from cultures growing at temperatures ≥ 38°C. An amount of 7.0 % ethanol (v/v) was obtained when 10% glucose was fermented by this yeast at 42°C in YPD medium. The same amount of ethanol was obtained when molasses containing 10% total reducing sugar was fermented for 12 hours at 42°C in a co-culture of *S. cerevisiae* and *I. orientalis*. This co-culture was inoculated to obtain an initial biomass concentration of 10 g. L-1. Thus, it seems possible to use this yeast to ferment hydrolisates of sugarcane bagasse when added to molasses or sugarcane syrups.

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PRETREATMENT OF SUGARCANE BAGASSE TO ACID OR ENZYMATIC HYDROLYSIS APPLYING THE ADVANCED OXIDATION PROCESS BY IONIZING RADIATION TO ETHANOL BIOFUEL PRODUCTION

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Figure 1. Sugarcane bagasse irradiation in batch system at the Electron Beam Accelerator in the Nuclear and Energy Research Institute, IPEN. (Photo Jesus Carlos/Imagemlatina)

The main objective of the project is to study the cleavage of lignocellulosic material from sugarcane bagasse using ionizing radiation from an industrial electron beam accelerator, in order to make easier the cellulose hydrolysis and the fermentation of their sugars to ethanol biofuel production. Sugarcane bagasse generally contain up to 45% glucose polymer cellulose, much of which is in a crystalline structure, 40% hemicelluloses, an amorphous polymer usually composed of xylose, arabinose, galactose, glucose, and mannose and 20% lignin, which cannot be easily separated into readily usable components due to their recalcitrant nature. The main difficulty to produce ethanol biofuel from this biomass is to break cellulose down into starches and sugars suitable for fermentation. The reactive species generated by the interaction of ionizing radiation with water (oxidant OH radical and redutants e-aq, and H radical) reveal as a very efficient way for the organic compounds oxidation in simple molecules and to enhance the processes of lignocellulosic enzymatic or chemical attack as well as the direct fermentation. The radiation effects on cellulose properties have been studied extensively, and the results have shown a decreasing of the polymerization degree and an increasing of the carbonyl content. Although this subject has been studied before, the sugarcane bagasse usually used were dehydrated or very old, and, in most cases, the cellulose was separated from bagasse before the radiation processing. In the present study it was used bagasse samples collect directly from the sugarcane mill. These samples have about 50% of humidity and the ionizing radiation does not change this parameter, which is a positive point for combination with enzymatic or chemical hydrolysis. The main challenge is to obtain the desirable effects applying doses as low as necessary to get some break in the polysaccharides, and at the same time to avoid the glucose loosing due to uncontrolled degradation of cellulose and hemicelluloses.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The irradiation of the sugarcane bagasse was carried out using Electron Beam Accelerator from Radiation Dynamics Inc., USA, with 1.5 MeV, and 37 kW from Radiation Technology Center of IPEN. It was applied absorbed doses from 5 to 200 kGy and then the bagasse samples were characterized by analysis of lignin, hemicelluloses and cellulose using standard methods. The enzymatic hydrolysis were done in the Sugarcane Technology Center, using a commercial Trichoderma reesei cellulase preparation (Celluclast 1.5 L), kindly supplied by Novozymes (Bagsvaerd, Denmark), with 5 FPU/g of cellulase and Beta-glycosidase 0.5% (p/p). The electron beam processing changed the sugarcane bagasse structure and composition and also caused some lignin and cellulose cleavage without loosing of sugar, with absorbed doses from 5 to 100 kGy. The conversion rate of cellulose to glucose increased from 8% to 14%, and from 6% to 18% in the Assay A and B, respectively. These represent 75% and 300% of yield after irradiation with 20 kGy of absorbed dose. There was a break in the lignin structure with doses lower than 50 kGy which was observed by the increasing of 20% of low molecular mass carbohydrates. Glucose and arabinose were liberated by the total cleavage of cellulose and hemicelluloses, respectively, when absorbed doses higher than 100 kGy were applied. The decreasing in the conversion rate above 30 kGy could be caused by the enzyme inhibition by some compounds e.g. furfural and 5-hydroxyl-methyl-furfural (HMF) that are formed during the saccharification of lignocelluloses polysaccharides and by the degradation of glucose. All the publications found in the literature are based on results obtained for doses higher than 200 kGy, that become the technology not feasible economically. The most important contribution of this study is the demonstration of promissory results with lower doses.

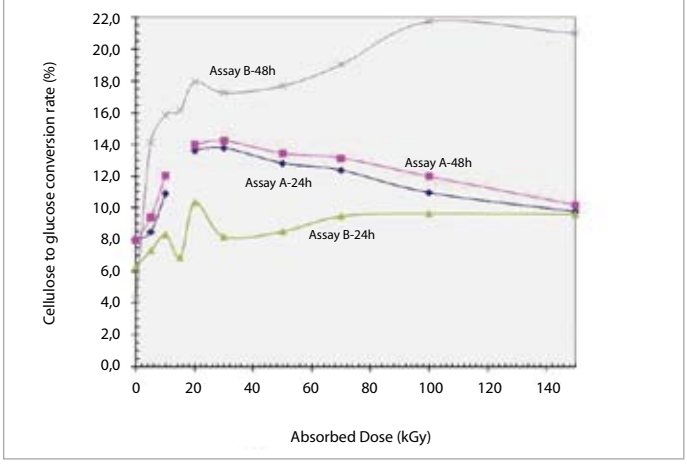

Figure 2. Conversion rate of cellulose to glucose in sugarcane bagasse samples from Assay A and B, related to the absorbed doses after irradiation at the Electron Beam Accelerator and enzymatic hydrolysis (24 and 48h)

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CATALYSTS FOR GLYCEROL HYDROGENOLYSIS: PRODUCTION OF GLYCOLS FROM BIOMASS DERIVATIVES

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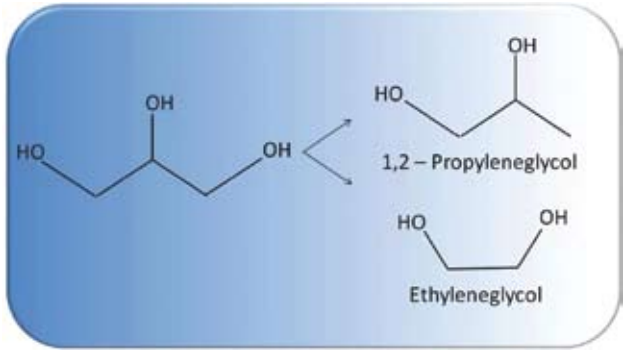

Figure 1. Hydrogenolysis of glycerol producing 1,2-PG and EG

The conversion of biomass and other renewable sources to higher-valued chemicals is one of the strategic goals of the 21th century. It is also a requirement concerning the challenges to be overcome related to environment and energy. Glycerol, a major byproduct of biodiesel production, is one of the main examples of low-cost, large-volume market product that can be used as a starting material for chemical transformation. The "glycerochemistry", which search for routes to process glycerol into more valuable commodity chemical, has become an intense area of research. An industrially relevant route involves the hydrogenolysis of glycerol to 1,2-propylene glycol (1,2-PG) and ethylene glycol (EG) (*Figure 1*). Both chemicals are widely used for manufacturing important products, from fibers to antifreeze and pharmaceuticals. In the hydrogenolysis reaction, hydrogen gas reacts with glycerol in the presence of a catalyst in several coupled steps, producing intermediates that are finally converted to EG or 1,2-PG. It is a complex reaction that is usually carried out under high pressure of hydrogen (500-1050 psi), high temperature (180-2500C) and for long reaction times (4-20 h), and alternatives routes and optimized catalysts are still desirable for large scale applications. As part of the partnership between Brazilian Synchrotron Light Laboratory (LNLS) and OXITENO S/A, the presented project co-funded by FAPESP aims the development of selective catalysts for hydrogenolysis of glycerol to glycols and the comprehension of the physicochemical characteristic that determine their performance. One of the main parameters guiding this work is the optimization of the selectivity to EG or 1,2-PG in a batch reactor, taking into account requirements for scaling up process. Studies on model systems and reactions supported by advanced characterization of catalysts using synchrotron techniques and electron microscopies will help to speed up the understanding of the system.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The control of glycol selectivity to 1,2-PG or EG requires the deep understanding of the reaction mechanism behind the glycerol hydrogenolysis. Although several works can been found on maximizing the selectivity for 1,2-PG [1] only in the last couple of years a good compromise between conversion and selectivity to EG has being achieved [2] . The main drawback is the uncontrolled C-C bond cleavage that may lead to a high percentage of gas products. For 1,2-PG production, Cu-based catalysts have presented good results while for EG production, Ru/C, transition-metal carbides and Ni-Raney are the most promising alternatives.

In the first year of the project the main effort was focused on the installation of the infrastructure required for catalytic tests. The assembled catalytic unit is composed by a batch reactor (Parr Instruments) of 300 ml, maximum operation pressure of P=1900 psi, temperature T=3500C and stir rate R= 1800 rpm, fed by H_2 from a P-controlled tank. The parameters of the reactor (P, T, rpm) as well as the pressure of the H_2 tank can be controlled remotely. For reaction products analysis we installed a gas-chromatograph (GC) equipped with FID and TCD detectors. The whole system was project following the set-up installed at OXITENO to promote a routine exchange of information and experience. *Figure 2a* shows the catalytic unit installed at LNLS.

Different catalysts have already been obtained and tested, such as Ru/C, Ni/C, Ni-W_2C and Ni-Raney. A general trend is the tendency for 1,2-PG formation among the liquid products after several hours of reaction. Selectivity to EG has been maximized only at short times and low conversions. *Figure 2b* shows preliminary results for Ni-Raney catalysts. This is in agreement with the main challenge to high production of EG that is to control the excessive cleavage of C-C leading to gas products. Next steps involve the detail analysis of the gas phase products, optimization of reaction conditions using concentrate glycerol solution (closer to industrial need), exploration of model reactions and improvement of Ni-W_2C catalyst synthesis.

a)

b)

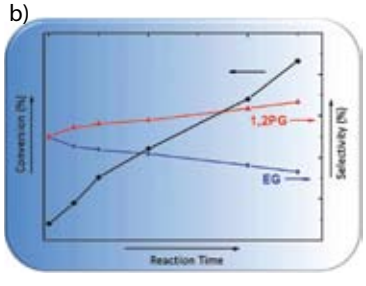

Figure 2. (a) Catalytic unit at LNLS and (b) preliminary results obtained with Ni-Raney catalysts

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INTEGRATING PHYSIOLOGICAL, MORPHOLOGICAL AND ANATOMICAL TRAITS TO UNDERSTAND THE DIFFERENTIAL SUCROSE YIELD IN SUGARCANE GENOTYPES

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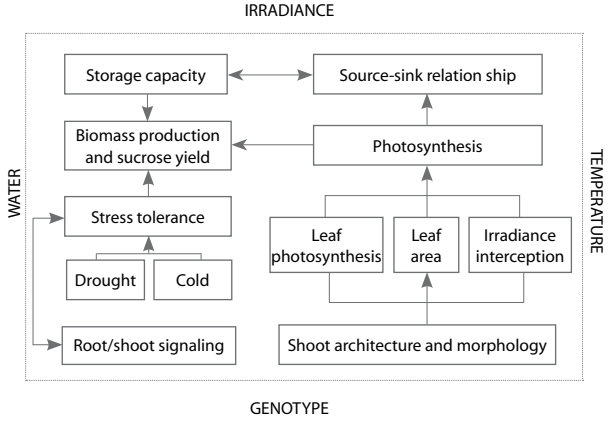
Our purpose is to understand some aspects of sucrose yield by addressing physiological, morphological and anatomical traits. This strategy will increase the subjacent knowledge about sucrose yield and let us to a more complex scenario about this important agricultural theme. In fact, improvement in the understanding of ecophysiological aspects related to phytomass production and sucrose yield of sugarcane is an essential condition to the development of the Brazilian sugar-ethanol sector. However, little is known about the relationship between plant traits and sucrose yield in the Brazilian sugarcane genotypes, an important issue for sugarcane breeding programs and modeling.

The following questions are relevant for the Brazilian agriculture, mainly considering that sugarcane breeding programs have periodically launched many productive cultivars: why do sugarcane genotypes accumulate differential sucrose amount in stalks? Is it a physiological and/or morphological and/or anatomical matter? Is it related to the source, sink or source-sink characteristics? Is the high sucrose yield related to differential sensitivity of sugarcane genotypes to stressful conditions found during the winter season? Is the differential sensitivity found in a specific phenological stage or in entire crop cycle? About those questions, an integrated approach for studying sugarcane plants is essential to study plant growth and sucrose accumulation. This challenge will be addressed in sugarcane genotypes with differential sucrose yield and canopy architecture growing under field and controlled (greenhouse and growth chamber) conditions. Several physiological, morphological and anatomical traits related to photosynthesis and sugar metabolism will be evaluated, with this project being the first step towards an integrative and interdisciplinary approach to understand the ecophysiology of Brazilian sugarcane genotypes. A team of experts from several well-recognized Brazilian institutions is prepared to deal with biomass production and sugar yield under a holistic point of view.



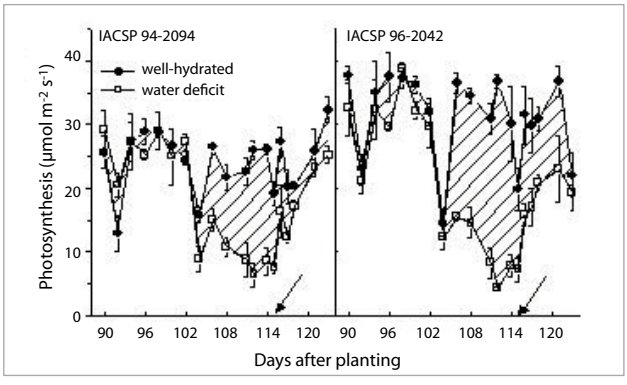


Figure 1 (results). Time-course of leaf photosynthesis as affected by soil drought. Arrow indicates re-watering

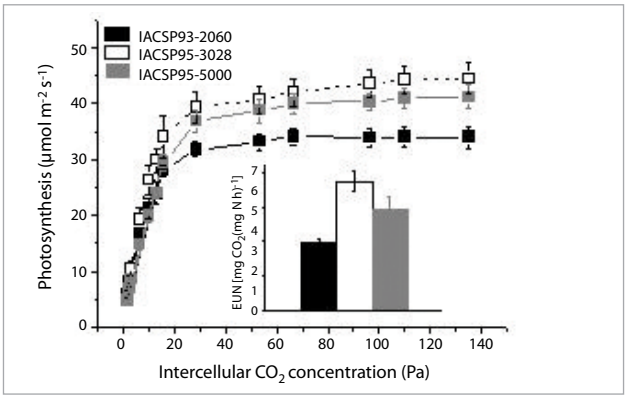


Figure 2 (results). Leaf photosynthetic response to increasing CO_2 concentration and nitrogen use efficiency in sugarcane varieties

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

We noticed significant variation on photosynthetic performance among sugarcane genotypes subjected to water deficit. Under such condition, differences on biomass production and sucrose content were also found when comparing plant genotypes. As compared to IACSP96-2042, the genotype IACSP94-2094 has anticipated stomatal closure in response to water deficit, maintaining higher leaf water potential when such stressful condition happened at the initial development phase. This phenological phase was the most susceptible to the deleterious effects of water shortage, regarding plant biomass of sugarcane genotypes. Besides a rapid stomatal response to soil drying, IAC94-2094 also exhibited higher stomatal conductance during water withholding period. As a consequence, higher leaf CO_2 assimilation and less reduction in plant biomass production were found in IACSP94-2094. Accumulation of leaf soluble carbohydrates – not sucrose – was also related to the tolerance of IAC94-2094 under constraining condition. Under water deficit, stalk mass was reduced about 11% and 49% in IACSP94-2094 and IACSP96-2042, respectively. An important issue regards the previous classification of such sugarcane genotypes in relation to drought response, in which both were considered tolerant according to plant yield under constraining environments. Our data confirmed such assumption for IACSP94-2094 and revealed that IACSP96-2042 is a productive genotype and has not drought tolerance from the physiological point of view. The higher sucrose yield and biomass production of IACSP96-2042 is probably related to its high photosynthetic activity when there is soil water availability. Regarding the canopy photosynthesis, we have found some interesting data. Significant differences in photosynthetic capacity were noticed among genotypes IACSP93-2060, IACSP95-3028 and IACSP95-5000, which are caused by mesophyll limitations. Although variations in potential photosynthesis have been found, environmental conditions restricted sugarcane photosynthesis, with genotypes showing similar values of diurnal CO_2 uptake at the superior canopy layer. On the other hand, the inferior canopy layer had an important role on photosynthesis of sugarcane plants, which is regulated by irradiance availability. High irradiance at the inferior canopy layer of IACSP95-3028 caused increased photosynthesis and improved plant vegetative development, given by increases in tillering, leaf area and accumulation of leaf and stalk phytomass. After 170 days of plant harvest, the sugarcane variety IACSP95-3028 with higher canopy biomass production also exhibited higher photosynthetic capacity at the superior canopy layer and higher diurnal CO_2 uptake at the inferior canopy layer when compared to IACSP93-2060 and IACSP95-5000. Another important result regards the nitrogen use efficiency, with the genotype IACSP95-3028 presenting the highest efficiency as compared to IACSP93-2060 and IACSP95-5000.

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LIBRARY GENERATION FOR BIOMASS-CONVERSION ENZYMES FROM SOIL METAGENOME

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Figure 1. Samples were collected from locations where plant biomass degradation is intensively occurring. Our first choice for collection of environmental samples was at a sugarcane field after harvesting. Because covering the soil with straws, it is expected that microbial population involved on lignocellulose degradation is enriched (photo by Luiz P. Jutter - CTBE)

The gradual shift from petroleum to renewable biomass resources is generally seen as an important contribution to the development of a sustainable industrial society and the effective management of green house emissions. Lignocellulosic materials, such as agricultural and forestry residues, are an abundant and low-cost source of stored energy in the biosphere. Thus, biomass conversion into feedstock sugars has moved towards the forefront of the biofuel industry. However, the saccharification of plant biomass is a complicated and lengthy process, mainly due to the inherent recalcitrance and the complex heterogeneity of the polymers comprising plant cell walls. Lignocellulosic biomass must go through an intensive pretreatment step, after which enzymes are used to break down the polysaccharides biomass into simple sugar suitable for fermentation and ethanol production.

Likewise, enzymatic conversion of cellulose and hemicellulose into simple sugar is also a demanding task, where a consortium of enzymes is needed for complete saccharification of these polysaccharides. Aiming at the entire exploitation of the plant cell wall polysaccharides, as an environmentally renewable energy source, an extensive repertoire of hydrolytic enzymes would play a major role for the success of this endeavor towards biofuel production. The objective of our effort is the generation of a toolkit of lignocellulolytic enzymes with a wide range of biotechnological applications, including their use as players for the development of strategies for second generation ethanol production. The prospection of these enzymes will be done from soil metagenome, which contemplates a pioneering strategy towards the prospection of biomass conversion enzymes from micro-organisms not conventionally cultivable. Additionally, this study may contribute to the development of the field of bioenergy by improving techniques for characterization of enzymatic hydrolysis and implementing heterologous gene expression in filamentous fungi.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The biotechnology has a continuous demand for novel genes, enzymes and compounds, and, so far, natural diversity has been the best supplier for these novel molecules. It is well known that in spite of the vast dataset of enzymes and microbes involved on plant biomass conversion, already described in the literature, it not been discovered yet a super microorganism that is capable of rapidly and efficiently degradation of all components of plant cell wall. Additionally, it is now widely accepted that the application of standard microbiological methods, for the recovery of microorganisms from the environment, has had limited success in providing access to the true extent of microbial diversity. As a consequence, the majority of the microbial genetic diversity (collectively known as metagenome) remains unexploited.

A consortium of microorganisms and enzymes is need for complete saccharification of the plant biomass in natural environment. Our study is focused on the prospection and characterization of the enzymatic system present in difficult-to-culture microbes inhabiting the soil. This study will contribute to the progress of the field of bioenergy by focusing: (1) the generation of library for biomass-conversion enzymes from soil metagenome, (2) improvement of techniques for characterization of enzymatic hydrolysis using capillary electrophoresis and scanning electron microscopy and (3) development of heterologous gene expression in filamentous fungi.

The generation of a library of biomass conversion enzymes, made through heterologous expression, presents a great potential of finding the best cocktails for lignocellulose degradation. Additionally to the wide-ranging industrial applications for these toolkit of hydrolases, the availability of purified celullolytic and xylanolytic enzymes shows importance as an analytical tool, not only for deciphering the fine structure of the cell wall architecture, but also for evaluation of required activities for a given pretreatment/enzymatic process for conversion of lignocellulosic biomass to environmentally friendly biofuels.

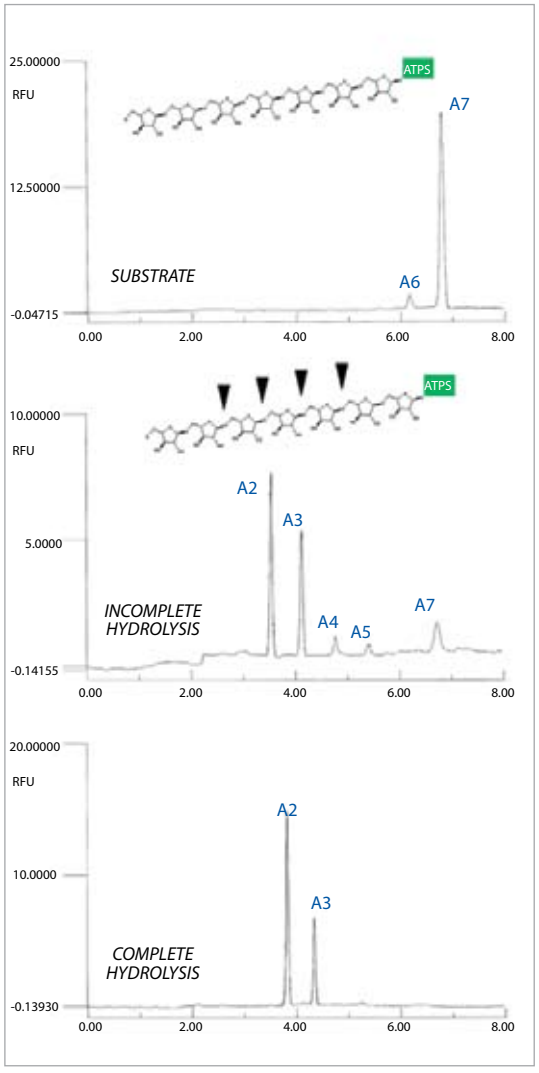

Figure 2. Capillary-zone-electropherogram of APTS-reducing-end-labeled-arabinoheptaose (substrate), intermediary (incomplete) and complete hydrolysis by endo-arabinanase from Thermotoga sp. CZE retention times of arabino oligomers, dimer (A2), trimer (A3) through heptamer (A7), and the predicted mode of operation of endo-arabinanase are depicted

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IDENTIFICATION AND CHARACTERIZATION OF microRNAS AND THEIR TARGETS IN SUGARCANE

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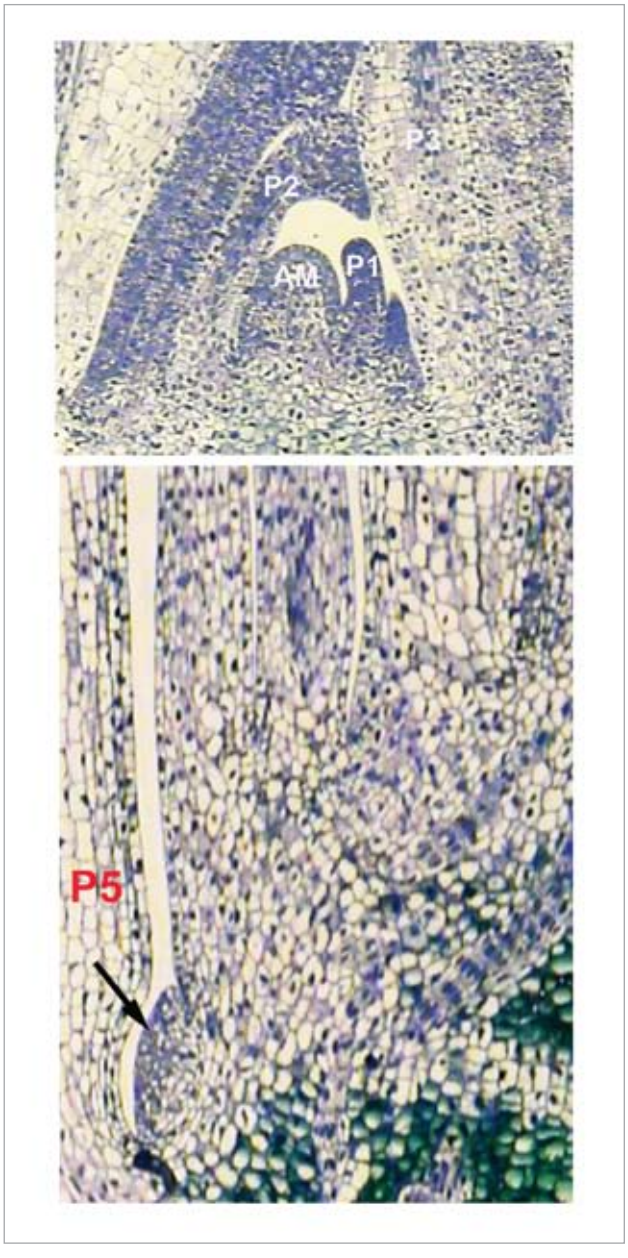

Figure 1. Meristematic tissues of sugarcane. The top panel shows the vegetative apex of a three-month-old S. officinarum *plantlet. The bottom panel shows an axillary meristem (arrow) of the same plantlet. AM: apical meristem; P1 to P5: examples of young leaf primordia*

Small regulatory RNAs and their targets form complex regulatory networks that control cellular and developmental processes in multicellular organisms. microRNAs (miRNAs) are a growing class of endogenous small RNAs that act in trans to regulate the expression of gene targets. miRNAs are processed from long, noncoding RNA polymerase II-dependent primary transcripts into mature miRNA (~21-24 nucleotides in size). Plant mature miRNAs and their targets frequently show near-perfect complementarity, facilitating their prediction using in silico approaches. Most of the known miRNA target genes are transcription factors that regulate critical steps during plant development.

The combination of cloning, deep sequencing and in silico approaches allows the discovery of conserved and species-specific miRNAs. Such approaches can also identify miRNAs that accumulate in specialized tissues/organs, such as apical and axillary meristems (*Figure 1*) as well as lateral buds. Members of some gene families involved in axillary meristem initiation and its further development are targets for regulation by miRNAs. Furthermore, transgenic and mutant plants overexpressing specific miRNA genes display increased number of branches/tillers as compared to wild-type plants. These findings suggest that miRNAs have important roles in this aspect of the development, which impacts the ultimate plant shoot architecture.

Shoot architecture is an important factor impacting biomass production and management practices for many crops, which are relevant characteristics for attractive biofuel crops. Although shoot architecture is to some extend influenced by environmental factors, it is determined mainly by the plant's genetic program that likely includes the action of miRNAs and their targets. Therefore, the identification and characterization of miRNAs involved in sugarcane plantlet emergence and development would increase our knowledge about the molecular controls of the establishment of plant shoot architecture.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

MiRNAs have been intensively studied in a wide range of plants over the past few years, but no systematic and comprehensive study has been performed on sugarcane, one of the most promising biofuel crop worldwide. To initiate our research, we searched for conserved sugarcane miRNA precursors using sequencing homology- and secondary structure homology-based strategies. Such strategies allowed us to retrieve several non-redundant miRNA precursors from EST and genomic survey sequence databases. The precursors were classified into 14 families.

Using an in-house BLASTn-based algorithm, we identified more than 40 potential non-redundant target sequences for the 14 sugarcane miRNA families. Consistent with the essential roles of miRNAs in regulating a variety of biological processes in plants, sugarcane target genes seem to be associated not only with development but also with diverse physiological processes. *In vivo* cleavage of some target transcripts was experimentally validated.

We subsequently evaluated the expression of selected miRNAs identified in distinct tissues/organs from commercial sugarcane hybrids as well as from two wild ancient progenitors (*S. officinarum* and *S. spontaneum*). Some miRNAs presented variations in abundance in organs/tissues of the hybrid as well as when comparing the same organs/tissues at similar developmental stages of *S. officinarum* and *S. spontaneum*. It is noteworthy that several tested miRNAs, though at variable levels, are expressed in lateral buds and apical meristems (*Figure 2*).

Functional studies may provide clues on the possible roles of these miRNAs in shoot architecture. We are currently testing this hypothesis by overexpressing microRNA precursors in sugarcane and model plants (such as *Arabidopsis* and *Brachypodium distachyum*). Additionally, we are generating small RNA libraries from dormant and active sugarcane lateral buds. This work would identify novel and species-specific miRNAs and other small regulatory RNAs associated with sugarcane bud outgrowth.

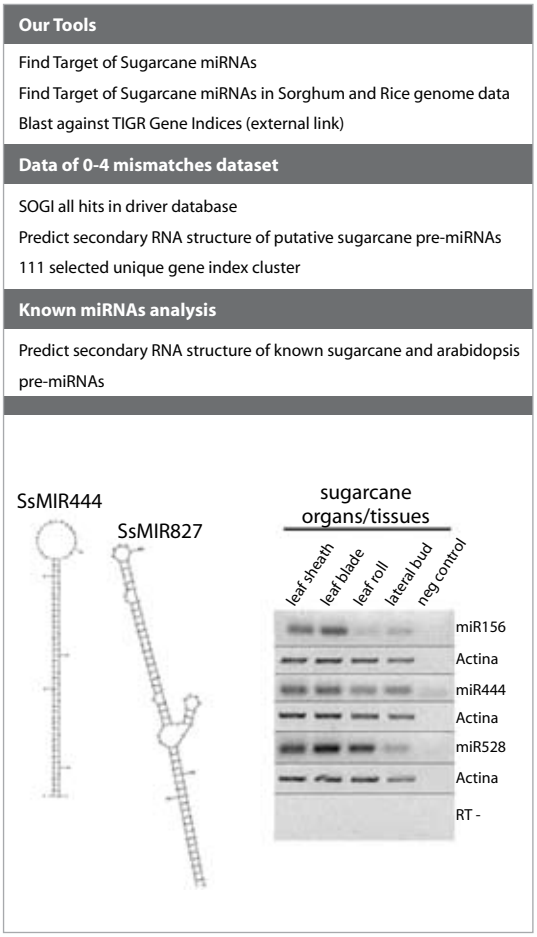

Figure 2. Sugarcane miRNA database (ScmiRbase). Top panel: web page containing the main search tools of the ScmiRbase. Bottom panels: examples of predicted secondary structures of sugarcane miRNA precursors and expression profiles of mature miRNAs in distinct organs/tissues

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A SYSTEM FOR LARGE SCALE PRODUCTION OF RECOMBINANT PROTEINS

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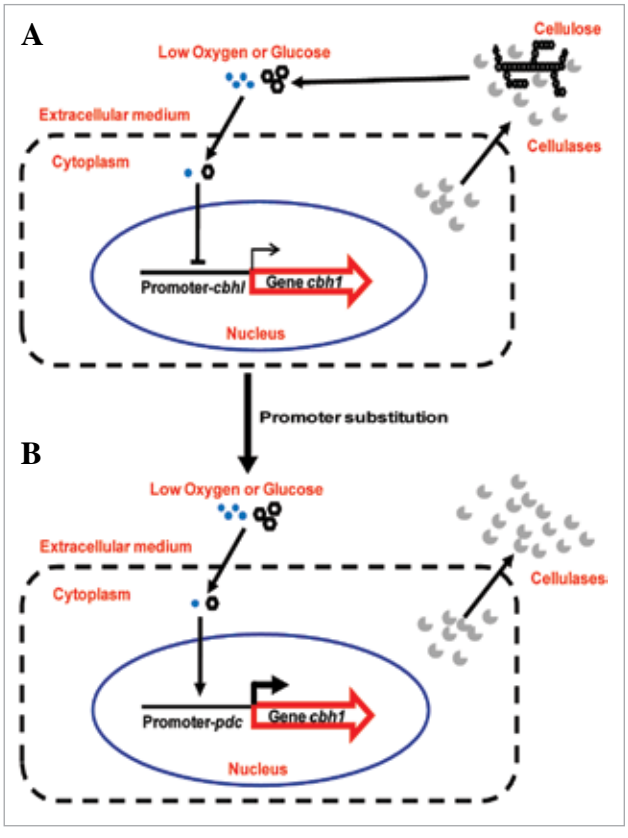

Figure 1. The cellulolytic system of T. reesei *requires induction by cellulose but is repressed by the cellulose degradation product – glucose, or low oxygen tension (A), however, substitution of the promoter (for example, of the cbh1 gene) for the pyruvate decarboxylase (PDC) promoter, which is strongly induced by glucose, or low oxygen tension (B), will be highly efficient to maintain the production of enzymes of industrial interest under repressing conditions*

In 2005, the Brazilian production of sugarcane bagasse summed 106,470 million tons. The utilization of this lignocellulosic residue for ethanol production is viable. It requires, however, a mixture of large amounts of enzymes essential for the hydrolysis of such residue to obtain fermentable sugars. The filamentous fungus Trichoderma reesei possesses an efficient secretory system that could be used in large-scale production of either homologous or heterologous proteins of industrial interest. Our proposal aims the construction of a system for large-scale production of enzymes by means of substitution or modification of *T. reesei* promoters capable of driving a large production and efficient secretion of enzymes involved in the degradation of biomass. The establishment of *T. reesei* mutant strains with prospective industrial use will allow the large-scale production of the necessary enzymes for biomass hydrolysis at a consequent lower cost, favouring the diffusion of biomass utilization as source for biofuels.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

In this project we aim to construct a system for large-scale production of enzymes through the genetic manipulation of *T. reesei*. We propose to substitute or modify promoters of this fungus (*Figure 1*) that have the capability of driving a highly efficient secretion of enzymes involved in biomass degradation. This would provide mutant strains with prospective industrial application, which would reduce cost and allow large-scale production of the enzymes required for the hydrolysis of lignocellulosic matter. Vectors bearing homologous or heterologous genes of enzymes involved in biomass degradation (cellulases, exo- and endoglucanases, glycosidases, etc.), under the control of promoters inducible at specific conditions (oxygen tension, concentration of glucose, or specific carbon source, etc.), will be constructed. One potential candidate is the pyruvate decarboxylase (PDC) promoter, which is strongly induced by glucose or low oxygen tension (*Figure 1*) (Chambergo et al., 2002; Bonaccorsi et al., 2006).

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SUGARCANE SIGNALING AND REGULATORY NETWORKS

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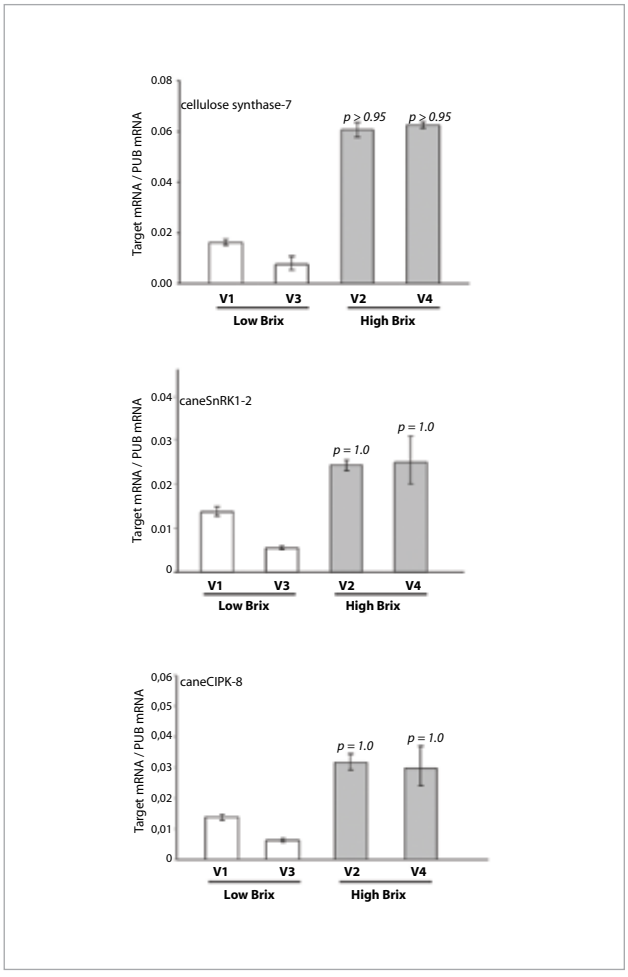

Figure 2. Cell wall metabolism and cell signaling genes associated with yield and sucrose content. Cultivars contrasting for biomass and sugar yield had their transcriptome profiled in search of genes that might be used as biotechnological tools for sugarcane improvement. The Figure shows qPCR experiments for 3 genes in two cultivars that are late in sugar accumulation and low biomass (V1 and V3) and two cultivars that are rich and precocious in sugar and biomass accumulation. (Waclawovsky, A. J., Sato, P. M., Lembke, C. G., Moore, P. H and Souza, G. M. (2010). Sugarcane for Bioenergy Production: an assessment of yield and regulation of sucrose content. Plant Biotechnology Journal. **8***:1-14.)*

We aim to study signaling and regulatory networks in sugarcane and to develop tools for a systems biology approach in this grass. As a starting point we intend to characterize three agronomical traits of interest: drought, brix and lignin content. We will study gene categories with a well known regulatory role (Transcription Factors, Protein Kinases and Phosphatases), conduct studies on the Transcriptome, produce transgenics, develop a database and computacional tools to integrate the several levels of information and we will initiate the whole genome sequencing of a brazilian sugarcane cultivar. In parallel, we intend to implement ChIP-Seq technology in sugarcane, to identify TF targets and gene promoters. The results will have multiple direct consequences on breeding programs that frequently select for CREs and TF changes in search for genotypes better adapted to the environment and with increased agronomical performance. PKs activate signaling cascades in response to environmental stimuli and our studies point to a predominant role of PKs in the regulation of sucrose content and drought responses. To identify new genes associated to brix, drought and lignin content we will characterize the transcriptome of genotypes and cultivars that contrast for these traits using olinonucleotide arrays. Genes of interest will be functionally evaluated by generating transgenics altered for their expression. To integrate the immense amount of public data and that generated by this project a robust computational infrastructure and database will be developed. The SUCEST-FUN database will integrate the SUCEST sequences, promoters, CREs, expression data, agronomical, physiological and biochemical characterization of sugarcane cultivars. We will also participate in the development of the GRASSIUS database to establish sugarcane, rice, maize and sorghum regulatory networks.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Modern sugarcane cultivars are complex hybrids with a giant genome resulting from crosses among several *Saccharum species*. Traditional breeding methods have been employed extensively over the years to develop improved varieties. Currently, commercial yields are at the range of 84 tons/ha/year. Our calculations of sugarcane yield potential leed us to believe that the theoretical maximum would be around 380 tons/ha/year (*Table 1*). Conventional variety improvement is limited by the narrow pool of suitable genes and the lack of biotechnological tools. Clearly, molecular genetics is seen as promising to assist in the development of improved varieties but a lot has to be done to bring sugarcane in par to other crops in terms of adequate technologies. Our group seeks to associate function to sugarcane genes using a variety of tools, in particular through the study of the sugarcane transcriptome and the production of transgenics. We developed customized oligoarrays representing 14,000 sugarcane genes. The arrays have been used to identify the transcriptome associated to yield, stress responses, sugar and cell wall metabolism, hormonal regulation and the circadian rythm of sugarcane plants. Overall we have conducted more than 300 hybridizations totalling over 40,000 thousand differential gene expression datapoints. An array of bioinformatic tools have been implemented and a database has been created that allow for storage and datamining. The SUCEST-FUN Database (http://sucest-fun.org) has been developed in the concept of the mediator approach that incorporates concepts from Data Warehouse and Federation and will allow for heterogenous data integration. A number of genes are being introduced into sugarcane plants and transgenics. Two of the genes have led to plants with increased sucrose content. The SUCEST-FUN Database assembles different databases such as the Sugarcane EST Database (SUCEST), signal transduction, transcription factors and metabolism gene catalogues which include expression data and records of the agronomic, physiological and biochemical characteristics of sugarcane cultivars. We have also started sequencing the genome of a brazilian cultivar and identifying gene promoters. Shot-gun sequencing using the Roche 454 Titanium plataform is underway and we have selected BACs enriched for genes of interest to help assemble this giant genome.

Table 1. Average, maximum and theoretical sugarcane yield and total dry matter production

Type of yield	Cane yield ($t\ ha^{-1}\ yr^{-1}$)	Biomass*	
		($t\ ha^{-1}\ yr^{-1}$)	($g\ m^{-2}\ d^{-1}$)
Commercial Average	84	39	10.7
Commercial maximum	148	69	18.8
Experimental maximum	212	98	27.0
Theoretical maximum	381	177	48.5

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FUNCTIONAL ANALYSIS OF THE TRANSCRIPTION FACTOR XLNR INVOLVED IN THE REGULATION OF TRANSCRIPTION OF CELLULASES- AND HEMICELLULASES-ENCODING GENES IN ASPERGILLUS NIGER

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Growth of the Aspergillus Niger *strain* N_4O_2 *át 30C in steam-exploded sugarcane bagasse*

The high cost of hydrolyzing biomass polysaccharides to fermentable sugars remains a major obstacle to be overcome before cellulosic ethanol can effectively be commercialized. The costs of cellulases and hemicellulases contribute substantially to the price of bioethanol. New studies to understand and improve cellulase efficiency and productivity are of paramount importance. Filamentous fungi like *Aspergillus niger* and *Trichoderma reesei* are impressive producers of hydrolytic enzymes already applied in a series of industrial processes, e.g. food, feed, pulp, paper, and textile industries. The *A. niger* xlnR transcription factor is a master regulator that activates enzymes of the xylanolytic system, a number of endocellulases and two cellobiohydrolases. The study of transcriptional regulators involved in the activation of genes that encode enzymes responsible for the degradation of cellulose and hemicellulose could provide several advantages for further genetical improvement of biomass-degrading microorganisms. A potential strategy to modify expression patterns of cellulase and/or hemicellulase could follow either a constitutive or induced expression of modified versions of the regulatory proteins. The design of constitutively activated or even structuraly modified transcription factors may lead to strains allowing inducer substance-independent enzyme production. Our project aims to provide basic information about the fine regulation at transcriptional level of *A. niger* genes that encode hydrolytic enzymes under the control of the transcription factor XlnR. Furthermore, we also plan to identify protein partners of XlnR that could be involved either in its down-regulation, for example via carbon repression, or additional proteins that help in the assembly of the transcription machinery aiming the establishment of the gene expression regulated by XlnR.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

We are currently evaluating mRNA accumulation by real-time RT-PCR for several genes encoding cellulases and hemicellulases, as well as establishing assays for enzymatic activity of cellulases and hemicellulases. We are deleting the gene encoding the catabolite repressor creA and overexpressing the gene encoding the transcriptional activator, xlnR. We are using sugarcane bagasse pretreated by steam explosion to evaluate the better enzymatic performance of these genetically modified strains.

Meanwhile, we are constructing strains with S-tag epitopes fused to these proteins aiming to identify protein partners involved in their post-translational regulation.

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NITROGEN NUTRITION OF SUGARCANE WITH FERTILIZERS OR DIAZOTROPHIC BACTERIA

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Nitrogen is required in large quantities for biomass production. Around 23% of the fertilizer N in Brazil is used in sugarcane. Besides being the most expensive plant nutrient, N fertilizers are an important component of the environmental budget of biofuel production. It is estimated that synthesis of N fertilizer accounts for about 25% of all the fossil energy spent in field operations for ethanol production from sugarcane in Brazil. Emission of nitrous oxide, a potent green house gas associated with fertilizer use, also adds to the environmental costs of ethanol.

There are evidences that biological nitrogen fixation (BNF) is responsible for supplying part of the N required by sugarcane plants because several diazotrophic microorganisms have been isolated in that crop. Besides, the amounts of N fertilizer applied to sugarcane in many cases do not replenish the N removed from the fields with the harvest or lost as part of management practices. However, old sugarcane fields generally do not show signs of soil degradation.

The actual contribution of BNF to sugarcane under field conditions is controversial. Some authors have expressed their view that BNF is of little relevance for sugarcane N nutrition. However, it is generally recognized that BNF presents great potential especially in Brazil where many studies have shown promising results.

BNF is affected by plant variety, bacteria species, and plant-bacteria interactions. An inoculant produced with five strains of N2-fixing diazotrophs was recently developed by Embrapa but it has not been extensively tested under field conditions. This project has the objective of studying the contribution of BNF to sugarcane production compared with the use of synthetic N fertilizer under different soils, environments, and sugarcane cultivars, evaluating the emission of N_2O from sugarcane fields fertilized with N, and testing an inoculant produced with endophytic bacteria. At the same time new N_2 fixing organisms are being searched for that are adapted to the sugarcane growing conditions of the State of São Paulo. The project will be complemented with studies on genetic traits of sugarcane associated with the capacity of N_2 fixation, which may help to obtain new varieties that can make better use of BNF.

Figure1. Field experiment with stalk seeds of sugarcane inoculated with diazotrophic bacteria. Blue tubes are the bases of chambers installed to allow the collection of green house gases, including N_2O, emitted from soil and N fertilizer. Gas sampling in the field

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Six experiments have been set up in the field and most results will be obtained after harvesting. One of the difficulties to assess whether the N in the plant came from fertilizer or BNF is that most of the nutrient taken up actually comes from the soil where it is continuously recycled. Besides, sugarcane plants in the field have already a natural population of diazotrophic bacteria that may overshadow the action of inoculated bacteria strains. Therefore, long term observations were planed for the accounting of partial inputs and outputs of N in the field, which includes the monitoring of plant yields and of soil N stocks in situations where zero or high N fertilizer inputs are used, so as to infer about any sizeable contribution of N fixation from the atmosphere. In addition the δ15N technique is being used to assess BNF by analyzing the isotopic composition of N species in sugarcane plants grown under field conditions.

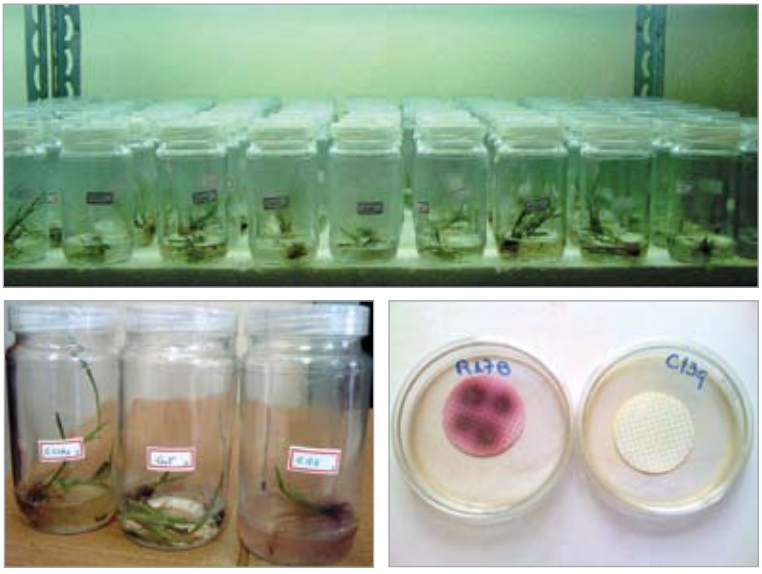

Figure 2. Above: micropropagated sugarcane plants inoculated with isolates of diazotrophic bacteria. Below left: diazotroph isolates growing in substrate containing sugarcane plants; Right: Isolate R178 showing indole production associated with plant growth promoting characteristics

Traits of different sugarcane genotypes associated with N_2 fixation will be characterized with molecular biology. *In vitro* tests are being carried out with several sugarcane varieties inoculated with the strain PAL-5 to evaluate their potential for BNF. At the same time the expression of genes involved with BNF and with the plant-bacteria interaction is being studied with the purpose of incorporating these characteristics in the sugarcane breeding program.

In order to broaden the community of known endophytic bacteria new isolations are being carried out. So far 160 isolates were obtained in one irrigated and one rain-fed experiment, from roots and stalks of varieties belonging to the breeding programs of Agronomic Institute, Ridesa, and Copersucar. Root colonization by diazotrophic bacteria was highly stimulated under irrigated conditions.

Eighty four percent of the isolates stood out as *in vitro* N_2 fixing organisms whereas 60% showed significant indole production. This latter characteristic is associated with phytohormone production that stimulate plant growth and development, which is another form of action of some of the diazotrophic bacteria. All isolates are being tested as to their capacity to promote growth of micropropagated sugarcane plants and, eventually to be used as inoculants.

If BNF can be managed in order to reduce the use of N fertilizer, either by inoculating plants in the field, or by learning the conditions that could favor N_2 fixation or still by breeding plants that can best benefit from BNF, the impact for bionergy production from sugarcane will be of great economic and environmental significance.

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FUNCTIONAL GENOMICS OF PHOTOSYNTHETIC GENES OF SUGARCANE

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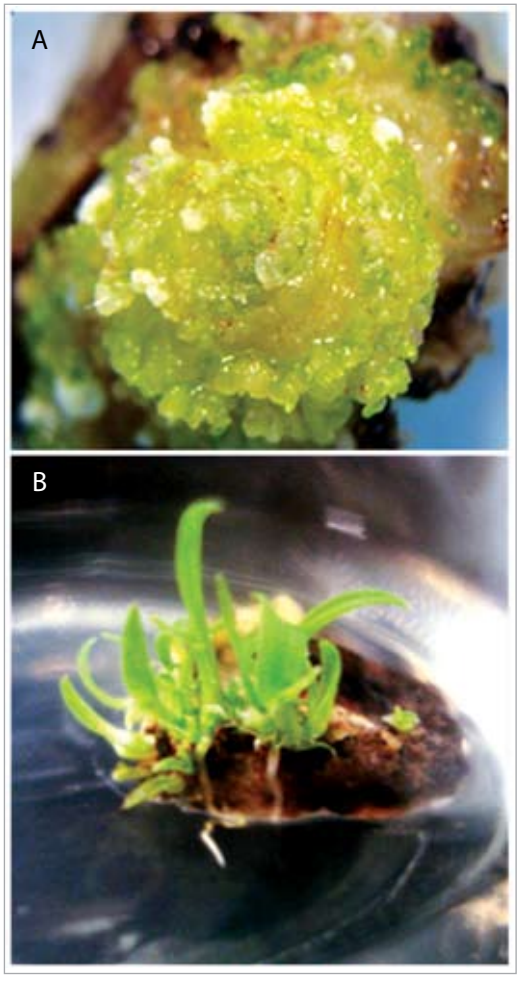

Figure 1. Regeneration of transgenic sugarcane; (A) Somatic embryogenesis from immature leaf-disc; (B) Leaf-disc with transgenic sugarcane

Sugarcane (*Saccharum officinarum*) is one of the most important feedstock sources for biofuel. In Brazil, sugarcane has been a prominent cultivated species undergoing accelerated expansion. This crop has launched Brazil as the major and most relevant country for exporting ethanol, as well as it became an important source of world bioenergy. In order to sustain and develop this enlarging agricultural and commercial sector, in a long-term, it becomes mandatory a continued quick release of increasingly productive sugarcane cultivars carrying specific advantageous traits, including increased sucrose content. Adversely, the breeding of sugarcane has been naturally limited by its low fertility, complex genome, narrow genetic basis, and long periods of 12 to 15 years to create a new variety. The development of efficient systems of molecular biology and genetic transformation are fundamental, and often the only way, to rapidly introducing new valuable agronomic and commercial traits into sugarcane elite germplasm.

Increase of sucrose content in elite sugarcane cultivars may be a main point to be addressed by using genetic transformation, and is directly dependent of increasing photosynthetic efficiency. The vast majority of photosynthetic proteins is nucleus-encoded and require N-terminal presequences, named chloroplast transit peptides, to target them to the chloroplast. About 2100 to 3600 distinct chloroplast proteins are nuclear-encoded, while about 100 to 120 are encoded by the organelle genome. The present project aims to develop efficient methods of sugarcane *in vitro* culture as well as methods of nuclear and chloroplast genetic transformation, applying them to modify photosynthetic genes in order to incorporate new photosynthetic traits in already productive Brazilian cultivars. The sugarcane photosynthetic efficiency is expected to be improved upon manipulation of photosynthetic genes (i.e. ribulose-1,5-bisphosphate carboxylase/oxygenase, phosphoenolpyruvate carboxylase, carbonic anhydrase) generating novel knowledge in this research field as well as leading to increased synthesis of triose phosphates and, ultimately, increased sucrose content in the transgenic cultivars.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Genomic and genome sequencing bring significant advances in chloroplast research to understand how chloroplast functions and communicates with other cellular compartments. The vast majority of chloroplast proteins are nuclear-encoded and require N-terminal pre-sequences, termed "chloroplast transit peptides", which target them to the chloroplast. Therefore, bioinformatics tools were used to identify genes associated with photosynthesis and with transit peptide sequences that, likely, are transported to the chloroplast for expression and function. Initially, we performed a keyword search for Arabidopsis chloroplast transit peptides in the UniprotKB database. Arabidopsis gene orthologous were then identified in sugarcane with TBLASTN using Arabidopsis protein sequences as queries against the SUCEST database. Around 650 sugarcane sequences with significant similarity (1e-10 e-value cutoff) were retrieved. The TargetP prediction of subcellular localization of the products of sequences showed that 245 are potentially targeted to chloroplast. In addition, we identified eight putative orthologous of known Arabidopsis and maize carbonic anhidrases (CA) by BLAST searches in sugarcane database using the most highly conserved regions of the CA amino acid sequences.

Concomitantly, we are establishing and optimizing direct plant regeneration and callus-based propagation methods in sugarcane. MS medium with different concentrations of 2,4-D and kinetin were tested to obtain highly embryogenic calli and to induce cellular dedifferentiation in the immature leaf discs prior plant regeneration. Results showed that immature leaf disc-based approach is a more feasible as well as cheaper and faster method to obtain directly plant regeneration as compared to embryogenic callus.

Genes associated with photosynthesis identified in the SUCEST database will be main targets to nuclear and plastid transformation. It is expected that analyses of these transgenic plants will shed light on sugarcane genetics, biochemistry and physiology and, furthermore, it is anticipated to accomplish significant improvements in specific agronomic and commercial traits within short time and at reduced cost.

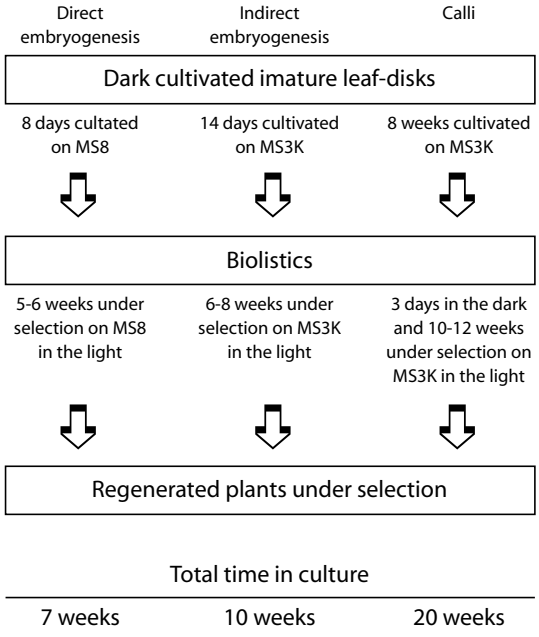

Figure 2. Relative timeframe to generate transgenic sugarcane plants from immature leaf-discs. Somatic embryogenesis was induced through three distinct regeneration processes: direct and indirect embryogenesis and calli

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STUDY OF THE TRANSFERENCE OF FIXED NITROGEN FROM DIAZOTROPHIC BACTERIA TO SUGARCANE

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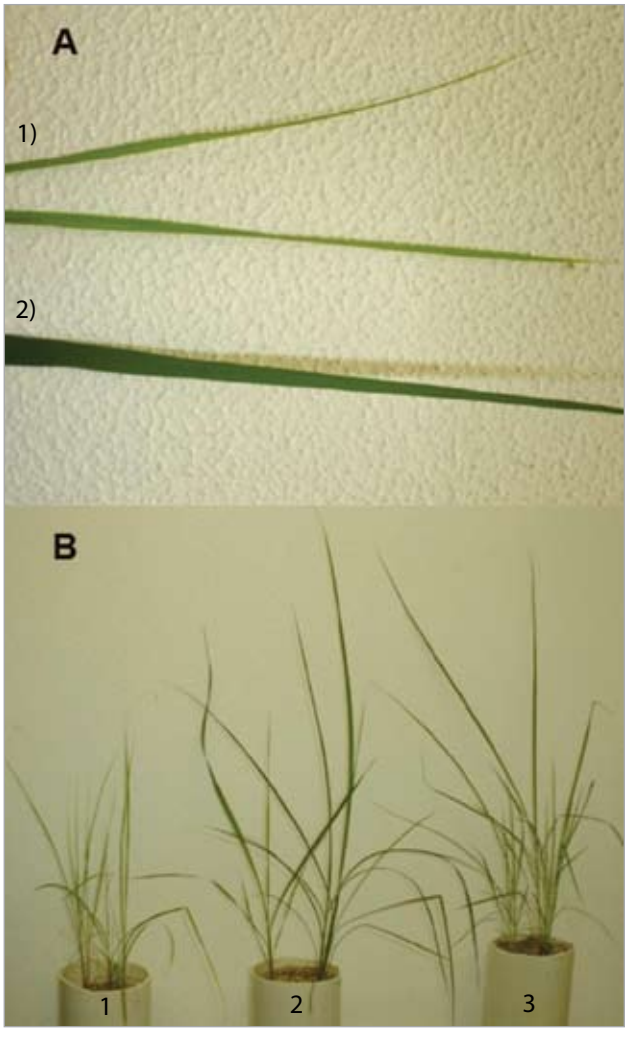


Figure 1. (A) Leaves of sugarcane plantlets treated in absence of combined nitrogen: 1) unfertilized; 2) submitted to organic fertilization. (B) Diferences between leaves lenght of sugarcane plantlets submitted to: 1) no fertilization (control); 2) conventional fertilization and 3) organic fertilization

Sugarcane is one of the most important crops in Brazil. This crop has a low response to nitrogen fertilizer, and even small inputs cause environmental impact. The use of biofertilizer as diazotrophic endophytic bacteria and organic fertilizer can decrease this impact. Endophytic bacteria live inside plant tissues and do not visibly harm the host. The influence of these bacteria on sugarcane is under investigation in this project.

Diazotrophic bacteria may play an important role in the nitrogen nutrition in sugarcane. The aim of the present project is to study the transference of fixed nitrogen from diazotrophic bacteria to sugarcane. To reach this objectives it will be performed: (1) analysis of the protein content and the C/N ratio in plantlets inoculated or not with endophytic diazotrophic bacteria isolated from sugarcane and submitted to different type of treatment: conventional, organic and control and (2) the evaluation of the possible interference of these bacteria on the nitrogen transport mechanism in sugarcane. (3) analysis of sugarcane callus grown in co-cultures with endophytic diazotrophic bacteria to study the bacterial interference on the callus proteins profile. Sugarcane callus can be used to evaluate if mixed diazotrophic bacteria can interact with each other and can also demonstrate the counter effect. The results obtained may contribute with knowledge on cultivation strategies. Thus, biodiversity will be used to the benefit of sustainable cultivation, evaluating the contribution of the nitrogen-fixing bacteria to the sugarcane, as well as the preservation of the soil and for the ecologic equilibrium.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

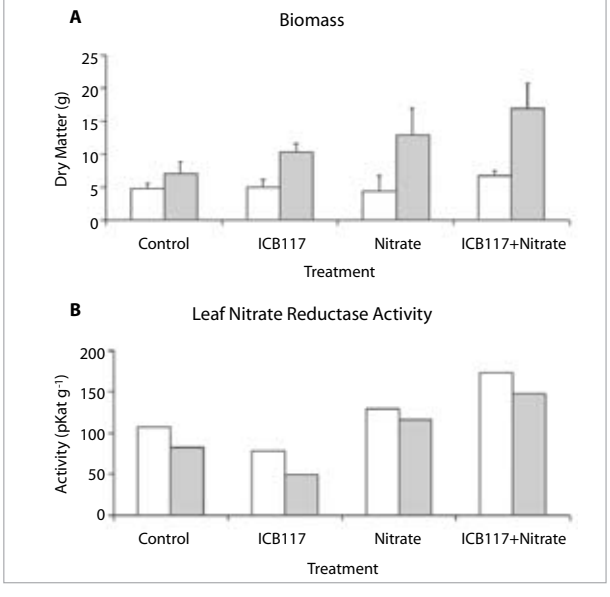
The treatments of sugarcane plantlets, after 60 days of incubation, showed that under organic fertilization, inorganic treatment and in control, respectively:
- a 3.0, 4.7 and 1.7 fold increase in the height of plantlets
- a 15.5, 31.0 and 3.4 fold increase in the fresh weight of plantlets
- a 25.5, 106.0 and 4.3 fold higher dry weight

Sugarcane plants were submitted to 4 treatments: (1) control (no inoculation and no combined nitrogen source); (2) inoculated with *Acinetobacter sp.* (ICB117) in absence of combined nitrogen source; (3) no inoculation, in presence of nitrate and (4) inoculated with ICB117 and in presence of nitrate. Bacterium Inoculated plants showed larger total dry matter, number of leaves and values of CO_2 uptake when compared to uninoculated plants submitted to the same nitrogen treatment. The enzyme nitrate reductase was more active in inoculated plants, in the presence of nitrate; in absence of nitrate, inoculated plants showed lower nitrate reductase activity than control. The endophytic ICB117 population was larger in plants treated without nitrate.

Using co-cultures, its was possible to evaluate that the influence of one bacterial genus on the callus depends on the bacterial strain; a mixture of two genera enhance the nitrogenase activity. Ongoing experiments, carried out in this project, aim to characterise these proteins and verify if there are differences between callus proteins in pure or in co-culture.

Future experiments will be carried out considering the presence or absence of inoculants and type of fertilization.

Figure 2: Dry matter (A) and leaf nitrate reductase activity (B) measured after 30 (white) and 60 (gray) days in plants under different treatments



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STRUCTURE AND FUNCTION OF ENZYMES AND AUXILIARY PROTEINS FROM TRICHODERMA, ACTIVE IN CELL-WALL HYDROLYSIS

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Lignocellulosic biomass, such as sugarcane bagasse, holds a promise of environmentally friendly bioenergy production in Brazil. However, enzymatic hydrolysis, currently considered a method of choice in biomass saccharification, is hampered by considerable cell-wall recalcitrance. To make this technology sustainable and cost effective, our comprehension of cellulose enzymatic hydrolysis should be significantly improved. Here we propose to conduct systematic structure-functional studies of *Trichoderma cellulases* and auxiliary proteins active in cell-wall degradation using a combination of X-ray protein crystallography, biophysical and biochemical studies, molecular dynamics simulations, statistical coupling analysis aligned with the site-directed mutagenesis and enzymatic assays aiming to obtain in-depth comprehension of cellulose hydrolysis. We plan to contribute toward structural analysis of *Trichoderma reesei* endoglucanases by solving a crystal structure of endoglucanase II (Cel5A), main enzymatically active, but structurally uncharacterized endoglucanase of this important industrial fungus. Moreover, we will contribute toward our knowledge of *Trichoderma cellulases* molecular organization by solving X-ray structures of main *Trichoderma harzianum* endo- and exoglucanases (primarily focusing on Cel7A and Cel5A) and by comparing them with the correspondent *T. reesei* enzymes. We also aim to structurally characterize swollenins, non-hydrolytic proteins, shown to enhance cellulose hydrolysis catalyzed by celulases, and to study thermodynamically its interactions with cellulose. In addition, we will construct chimeric enzymes by fusing of swollenin with the cellulases and will study enzymatic properties of such chimeras. Furthermore, we will conduct systematic molecular dynamics studies of the cellulases and swollenin, and investigate their flexibility by hydrogen deuterium exchange followed by mass-spectrometry. Finally, we will use all these acquired knowledge to modify the proteins using site-directed mutagenesis aiming to better comprehend molecular basis of their function and to produce enzymes and their mixtures with enhanced hydrolytic properties.

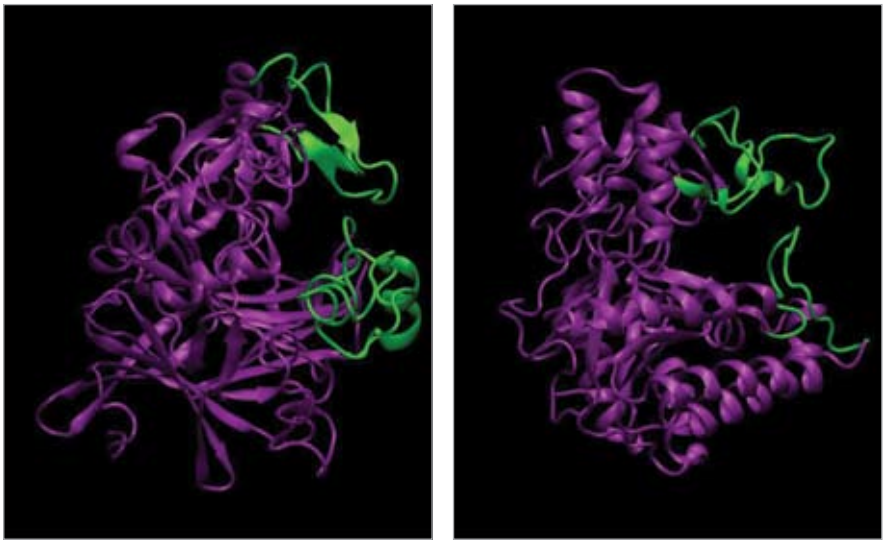

Figure 1. Catalytic core domains of T. reesei *CBH I (Cel7A, left) and CBH II (Cel6A, right). Loops in green highlight tunnel roofs. Tunnel lengths are 50 Å and 20 Å long (out of the plane of the paper) in CBH I (Cel7A) and CBH II (Cel6A), respectivelly*

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Enzymatic hydrolysis is one of the crucial steps in cellulosic ethanol production, for example from sugarcane bagasse or eucalyptus tree wood. The importance of this process steams from the considerable recalcitrance of biomass to saccharification procedures. To optimize cellulosic bioethanol production and to turn it cost-effective, we need to comprehend a process of enzymatic hydrolysis on the molecular level and therefore, to decipher structures and functions of the enzymes that participate in this process and to understand how main enzymatic components interact with each other during hydrolysis of biomass. As a first step in this direction we advanced with fermentation, purification and characterization of cellulases from the filamentous fungi *Trichoderma harzianum*, *Trichoderma reesei* and *Aspergilus niger* and their structural and enzymatic studies, as well as with structural studies of other hydrolytic enzymes, such as (*R. marinus laminarinase*, *T. reesei* beta-mannosidase, *Xantamonas citri* endoglucanase and lignine oxidases, among others). Our aims is to proceed with the structure-funcional studies of glicosyl hydrolases, to improve our understanding of their concerted action during the process of enzymatic hydrolysis and to contribute to the development of enzymatic blends with improved hydrolytic properties, particularly as applied to sugarcane bagasse and eucalyptus tree biomass.

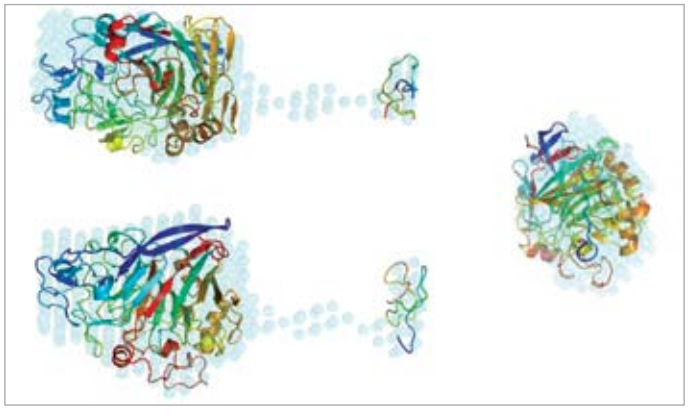

Figure 2. Superposition of small-angle X-ray scattering (SAXS) derived low-resolution envelope of T. harzianum *CBHI with two separate high-resolution structures: of the catalytic domain of CBHI from* T. reesei *and of its cellulose-binding module (CBM). Three orthogonal views are given*

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N_2O, CO_2 E CH_4 EMISSIONS FROM SOIL DURING AGRO-BIOFUEL PRODUCTION IN SÃO PAULO STATE, BRAZIL

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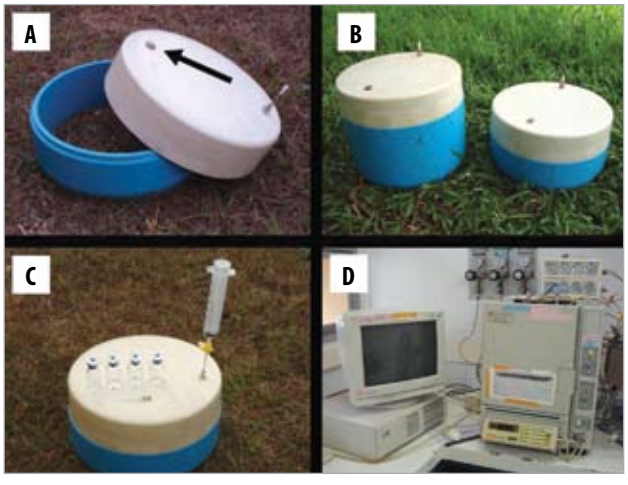

Figure 1. Chambers used for the collection of N_2O, CO_2 and CH_4 (A,B) showing flasks utilized for the storing the samples (C), details of the orifice that equilibrates the internal and external pressures of the chamber (A - arrow) and collection of the sample utilizing a 60 ml syringe (C) and gas chromatograph equipment (D)

Brazil is the world's largest producer of sugarcane, with an annual crop yield of over 470 x 106 metric tons in 2006-2007, planted in approximately 7 million ha. About half of sugarcane in Brazil is planted in the state of São Paulo, where sugarcane is the main agricultural product and contribute to about 27% of the state's GDP.

With about half of the global ethanol production, Brazil is already the largest contributor in the international ethanol trade. Yet, production is predicted to continue to expand due to geopolitical instability in oil producing countries and an increasing commitment from developed countries to the Kyoto Protocol to reduce emissions of carbon dioxide and other green house gases.

According to estimates from models and numerical analyses, Brazilian ethanol ranks among the best biofuels in terms of net energy produced for the amount of fossil fuel used in the production and, consequently, of CO_2 emitted. Also, sugarcane crops in Brazil grow with less nitrogen fertilizers than other biofuel crops, such as corn, which results in lower levels of nitrous oxide, a potent green house gas, during the production of Brazilian ethanol. However, the lack of real measurements and actual data about emissions of green house gases (GHG: N_2O, CO_2, CH_4) associated with the production ethanol in Brazil hinders our capacity to properly quantify its effectiveness at reducing emissions of GHG. Studies estimates that soil emissions of GHG, which are not associated with the consumption of fossil fuels, account for more than 50% of the total emissions. Meanwhile, *in situ* estimates of nitrous oxide ($N\text{-}N_2O$) emissions from fertilizer application in sugarcane fields in Brazil are in the order of 1%. If confirmed with further *in situ* measurements in a more comprehensive study, these low GHG emission can have important implications for the sugarcane industry in Brazil. In this project, we propose to determine *in situ* emission of GHG from soils, according methodology presented in *Figure 1*, planted with sugarcane in the state of São Paulo during its productive cycle to improve and expand existent estimates. *In situ* measurements of GHG in Brazilian sugarcane fields are practically non-existent, probably because N losses from fertilizers as N_2O ($N\text{-}N_2O$) are assumed to be insignificant in comparison to other losses, and because fossil fuel use during sugarcane production is low because much of the management practices in Brazil rely on manual labor. With eminent changes about to occur in the sugarcane ethanol industry in Brazil, these assumptions need to be revised and new data collected to guarantee the low emission.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

According to estimates of GHG emissions generated from the burning of agricultural residues in Brazil since 1994, sugarcane accounted for about 97% of the emissions. However, the lack of field data and measurements from different systems of agricultural production create large uncertainties in emission calculations.

In this project, we expect to produce a complete assessment of GHG emissions from soils in sugarcane crops in the state of São Paulo. By evaluating the variability of emissions as a function of management practices and climatic variation during measurements, we also expect to determine the hot spots and hot times for GHG emission during the sugarcane crop cycle so that strategic plans can be targeted to minimize these emissions.

Overall, we plan to produce reliable and realistic data on GHG emissions from sugarcane soils in São Paulo in order to calibrate and validate soil emission models that can be used to estimate emissions of GHGs from sugarcane plantations. By improving present estimates, predictions of the GWP of ethanol produced in Brazil can be properly assessed and compared to other forms of biofuels. We understand the complexity of representing the wide range of conditions for GHG emissions from the approximately 140 sugarcane mills in the state of São Paulo, where sugarcane grows under different management practices, climatic conditions, and soil types. However, by including different experimental treatments with a wide range of management practices and rates of fertilizer application and use of agricultural waste, we should be able to address the complexity of the system.

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ETHANOL APPLICATION AS FUEL: PLASMA IGNITION FOR VEHICLE ENGINES

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Figure 1. Spark plug electric discharge

Petroleum oil is an important source of energy and a raw material that has been widely exploited by mankind. There is a concern about its storage in nature, since it is a non-renewable source and will be exhausted in near future. The indiscriminate use of oil products produces serious consequences for environment, like carbon compound emissions. Therefore, it is necessary to develop strategies to minimize emissions of air pollutants. The replacement of fossil automotive fuels by alternative and renewable fuels is increasing in order to reduce emission of toxic gases in the atmosphere. In this sense, the ethanol fuel, in Brazil, was implemented through National Alcohol Program (Pro-Alcool). More recently, the National Laboratory of Science and Technology of Bioethanol (CTBE) was also created in order to ensure Brazil leadership on sustainable production of sugarcane and bioethanol through development and innovation. The ethanol is undoubtedly cleaner than gasoline due to less toxic emission substances, such as benzene and butadiene. Furthermore, by having a simpler composition, bioethanol releases lower levels of complex substances into the atmosphere during its combustion.

Ignition engines by spark discharges (or internal combustion engines) initiate their combustion mechanisms leaded through electrical discharges. For a fuel-air mixture ignites into an engine combustion chamber, an electric discharge which occur between the electrodes of spark plug, provides enough energy to the mixture to be completely burned, thus obtaining the maximum engine power.

This project focuses on the investigation of processes occurring during the ignition of plasma and its consequences in post-discharge for an internal combustion engine, especially considering the spark plug discharge (*Figure 1*), aimed at finding the proper parameters to be applied in cars that operate on "poor mixtures" reducing pollutants released into the atmosphere. The research aims is to point out methods and materials to be used in order to provide an analysis of the processes occurring in plasma and combustion.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

After the implementation of a synchronic circuit, we are able to generate controllable electrical discharges . These discharges were characterized in terms of electrical properties from a high resolution oscilloscope. Voltages and currents were measured according to the applied pulses. The voltage values were found to be around 6 kV for 40ns. However, the current values were found to be around 100 mA for 2.3ms (*Figure 2*). Through these data, it is possible to conclude that even without electric field, there still exist the production of ions and reactive species.

In collaboration with the Geophysics Space Division Group of National Institute for Space Research (INPE), the emission light during the break-down process of the discharges was recorded from a high-speed camera usually used to shoot lightning. From these data, the discharge light intensity was inferred as a function of time. Comparing the light curve with the normalized electric current graph (*Figure 3*), it is possible to check that the discharge duration is about 2.3ms.

By using techniques of emission spectroscopy, several discharge parameters, such as temperature, electron density and electron temperature will be obtained during the combustion.

The aim of our work is then to optimize these discharges in order to improve the burning of the bioethanol fuel inside a high-pressure combustion chamber and therefore to use a "poor mixture" to achieve good engine performance.

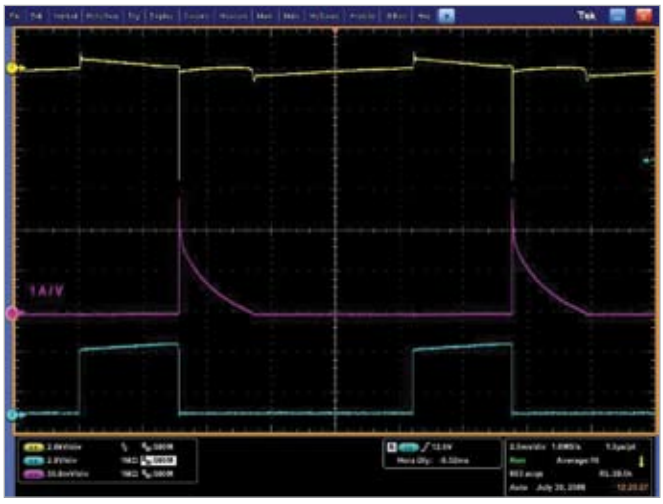

Figure 2. Curves of voltage (yellow) and current (purple) for the entrance pulse (blue) with 10ms and 30 % of duty cycle

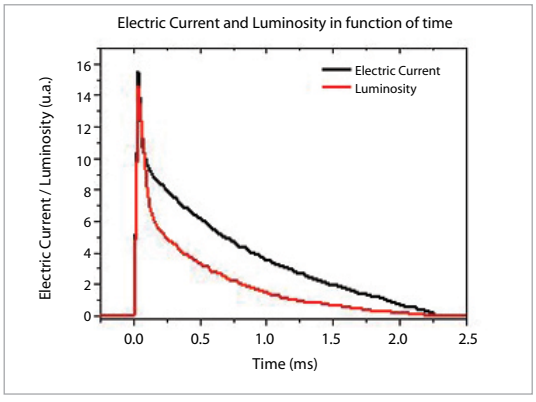

Figure 3. Curves of electric current and luminosity as a function of time

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ORGANIZATIONAL DESIGN OF BIOEN PROGRAM: INTELLECTUAL PROPERTY, INCENTIVE MECHANISM AND IMPACT EVALUATION

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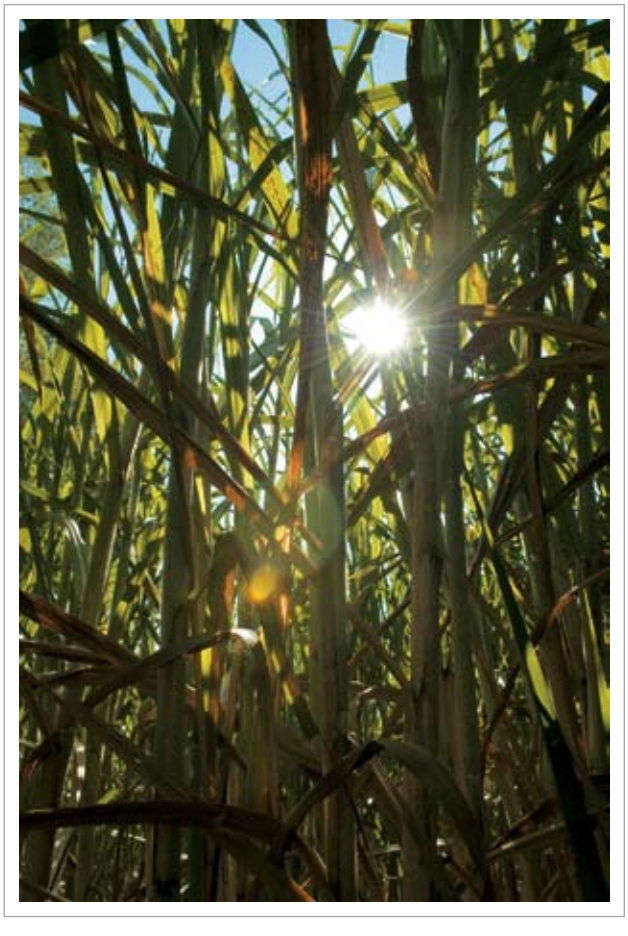

Photo by Eduardo Cesar

The project aims to attend one of the components of the call for the BIOEN Program related to intellectual property and technology transfer. It consists on a multidisciplinary project that articulates economic issues, assets management and social network formation together with scientific and technical components of the researches that are being selected to join the program of studies on Bioenergy.

The project's general goal is the formulation of an organizational design that involves the projects of BIOEN considering three basic approaches: a) analysis of the demands due to distinct forms of intellectual property of technologies, supplies and genetic material that can block or create risk situations for the continuity of specific projects or even of the program itself; b) preparatory analysis of business plans based on the construction of economic exploration 'models' of BIOEN research results in different levels, from intermediate products and supplies to final products, as, for example, new improved variety. It also includes the relation between different projects and an incentive system, and scenarios of partnership formation for products development.

The models must be built up from the formation of networks that identify patent families, networks of quotations and case studies on intellectual property attribution in vegetal biotechnology, focusing the bio energy field; c) a block of ex-ante impact evaluation that provides subsidies for the formulation of business plans based on the research results.

The project strategy consists in establishing a training program with four advanced undergraduate or graduate students and a trainee in computer sciences. Under de supervision of the project, they have to prepare training material and the collect subsidies from the Workshops to perform multicriteria analysis and generate scenarios in the three main areas of the project.

About ten Workshops are programmed for the 4 years project schedule, half of them with BIOEN project participants (scientists, technicians and managers) and 5 with external researchers and BIOEN staff looking forward building scenarios of property rights and evaluation of impacts.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The project started in September, 2009. Three meetings was hold by the group to the initial definition of the research tasks for the next four years and the strategy to keep the three groups connect alongside the research schedule.

The team, headed by the Institute of Economics of UNICAMP comprised: a) a team responsible of the ex-ante evaluation of the impacts of BIOEN Program in selected sectors of Brazilian Economy. It has 3 senior researchers, faculty at FEA/USP – Dr Abraham Yu has the role of coordinator of one research area – and a group of graduate students; b) a team in charge of FTO and MTA analysis, headed by Prof. Maria Beatriz Bonacelli, faculty of the Department of Technology and Science Policy of the Geosciences Institute, UNICAMP. It comprises three more senior researches from the Public Policy Institute/UNICAMP; Federal University of Grande ABC and a Pós-Doctor Researcher placed in the Geosciences Institute, sponsored by a CAPES program. A group of training students will be contacted to help development of the research; c) a Contract Design and Patent Evaluation and Scientific foresight group is located at the Institute of Economics. It is coordinated by Prof. José Maria F.J da Silveira, coordinator of the Project and Prof. Maria Ester Soares Dal Poz, faculty at Applied Sciences Institute of UNICAMP (FCA-Limeira), with the assistance of the Prof. Jacques Weiner, Institute of Computer Sciences, UNICAMP. A grant for training a graduate in Computer Sciences is planned by the research and the results are expected for the end of the year 2010. Undergraduate student with a grant for initial research sponsored by National Council for the Development of Research (CNPQ) is reviewing literature related to patent valuation.

Photo by Eduardo Cesar

Some contacts with research groups abroad, like University of Limburg University, Adelaide University (in Australia), and Wageningen University (at Haia, The Netherlands) and University of California at Berkeley are planned to be established in the year 2010, in the aim of gathering methodologies to sustain mostly the areas "b and c" mentioned above.

MAIN PUBLICATIONS

As mentioned above, the project is its very beginnings. Up till now there is no papers published based by the research team. It worth mentioned a that a paper related to Intellectual Property in Agriculture was presented by Adriana Pinto Vieira in the XIII Latin American Meeting in Technological Management, ALTEC, in November, 2009, in Cartegena das Indias, Colombia.

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IMPACTS OF THE EXPANSION OF THE SUGARCANE AGROINDUSTRY ON FRESHWATER COMMUNITIES

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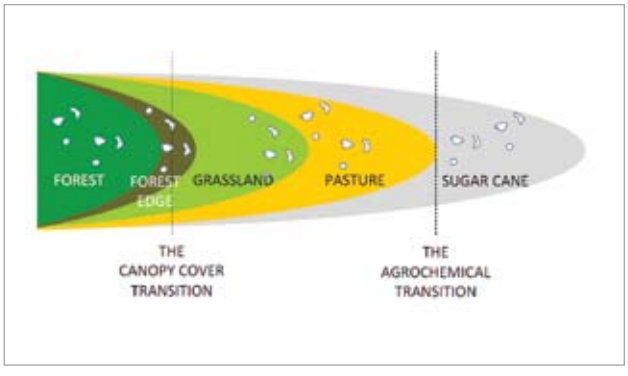

Figure 1. The gradient of environmental degradation in landscapes of sugarcane expansion ranges from native habitats (various cerrado physiognomies) to pastures and sugarcane plantations and is here conceptualized as presenting two steep transitions in physico-chemical properties, with important consequences for the organization of freshwater communities: the canopy cover transition, where most of the changes in the community are expected to be mediated by the presence or absence of a canopy and its influence on pond hydroperiod, temperature and primary production, and the agrochemical transition, where most of the changes in the community are expected to be mediated by the employment of fertilizers and pesticides and their influence on water quality

The dawn of a new paradigm in energy supply – biofuels – points to the continued expansion of agriculture in Brazil in the near future. The country is in a favorable position to assume the global leadership in biofuel production for possessing both ideal geographic and environmental conditions and the already most efficient ethanol industry worldwide. Not surprisingly however, agriculture involves both benefits and costs to society. Industrial agriculture is one of the most environmentally harmful human activities, being directly involved in habitat destruction and in the contamination of water resources. It is unacceptable that Brazil, entering the XXI century with the largest share of the world´s biodiversity and native tropical habitats, and with adequate technical and scientific human resources, misses the historical opportunity to assume, in addition, a model role in reconciling economic growth with environmental preservation.

This project proposes to test the hypothesis that the expansion of sugarcane has substantial impacts on freshwater communities, a significant part of which can be directly or indirectly attributed to agrochemicals such as fertilizers and pesticides. More than documenting impacts, it proposes to understand the mechanisms through which these impacts are generated. This project proposes in addition to validate, for tropical systems, methodologies employed in ecological and ecotoxicological studies in temperate systems, as well as to establish the foundations for the development of a bioindication concept for the contamination of water bodies. These objectives will be achieved in a broad research programme involving sampling and experimentation in laboratory, mesocosms and field. Sampling surveys of temporary pond communities – including algae, tadpoles and predatory insects – across a gradient of environmental degradation (*Figure 1*) will reveal patterns of association among land use, environmental physico-chemical properties, and community composition and structure. In turn, experiments will test the importance of agrochemicals in generating the observed patterns. Through studies conducted in multiple experimental scenarios, we aim at generating a line of extrapolation from lab to field, and to establish clear cause-and-effect relationships between hypothesized processes and observed impacts. Knowledge derived from this project will be important in the development of better agroindustrial practices, towards sustainability in biofuel production and a larger acceptance of Brazilian biofuels in international markets.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

As a first step towards understanding the potential hazards of agrochemicals, we reviewed the toxicity of all 225 pesticide formulations registered for use in sugarcane in Brazil and their potential to cause effects of concern for humans or the environment. Among the 62 active ingredients employed, we found one compound listed as banned or severely restricted in the Rotterdam Convention; two (possibly three) compounds included among the ´Dirty Dozen´, a selection of chemicals of priority concern that are generally persistent organic pollutants (Stockholm Convention); and 26 compounds considered ´Bad Actors´ by the Pesticide Action Network, substances that are highly toxic in acute exposure, neurotoxic, probable or known carcinogens, known groundwater contaminants, and/or of known reproductive or developmental toxicity. Regarding hazards specific to the aquatic environment, 16 compounds are of high or very high acute toxicity to the aquatic biota (*Figure 2*).

We recently completed two pilot sampling surveys in over 30 water bodies. These pilot surveys will provide the basis for associating land use, environmental physico-chemical properties, and freshwater community composition and structure in the definitive sampling surveys of 2010/2011. The experimental component was started with an evaluation of the toxicity of fertilizers to anuran larvae in both acute and chronic exposure in the laboratory. Focusing on the inorganic nitrogen species – nitrate, nitrite and ammonium – we investigated lethal and sublethal endpoints including effects on behavior, growth, and development. Nitrate, the most chemically stable and pervasive nitrogen species, is of lower toxicity when compared to nitrite and ammonium. As expected, lethality increased with prolonged exposure; in other words, concentrations considered harmless in the more traditional, standardized, short-term ecotoxicological bioassays could be lethal in a more ecologically realistic long-term exposure. The experience acquired is useful in the development of a tropical model experimental system. Some species but not others have been shown to be amenable to maintenance and experimentation in the laboratory from egg to metamorphosis, and as such have excellent tractability for ecological and ecotoxicological studies.

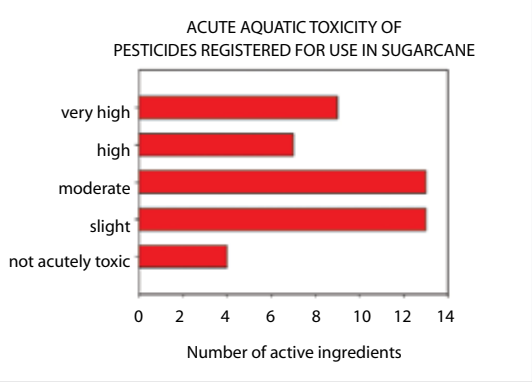

Figure 2. Distribution of the toxicity of the active ingredients included in pesticide formulations registered for use in sugarcane in Brazil to aquatic organisms in acute exposure. Among the 62 registered active ingredients there are also 38 known to be toxic to aquatic organisms in chronic exposure, as well as 8 known and 17 potential groundwater contaminants. Data from Schiesari and Grillitsch in press

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FUNCTIONAL ´OMICS OF THE RATOON STUNTING DISEASE OF SUGARCANE

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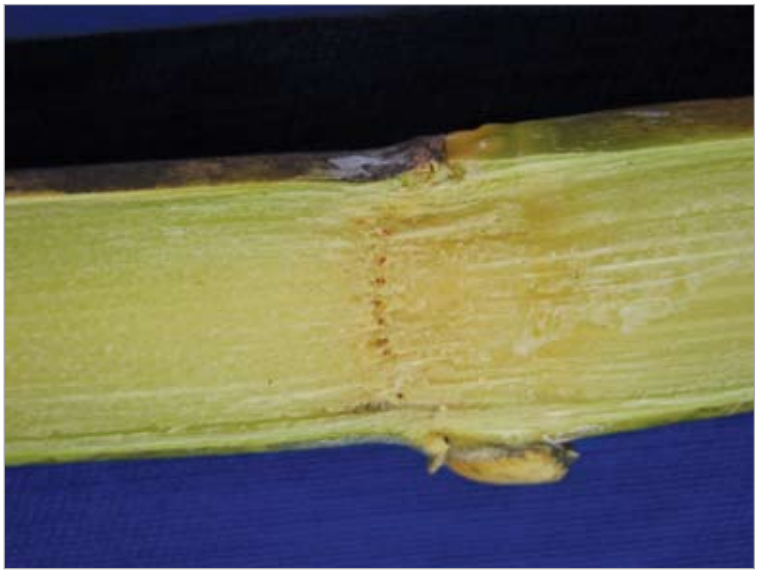

Figure 1. Internal symptoms caused by Leifsonia, characterized by small redish dots below the plant internodes. Infection by this bacterium is characterized by the absence of conspicuous external symptoms

The ratoon stunting disease (RSD) of sugarcane is caused by the fastidious xylem-limited gram-positive bacterium *Leifsonia xyli* subsp. *xyli* (Lxx). RSD is one of the most important diseases of sugarcane worldwide. Although control of the bacterium relies primarily on using healthy heat-treated stalks as planting material, this approach is not 100% effective and, given the perennial nature of sugarcane plants and the prevalent mechanical mode of transmission of the bacterium, the disease can reach epidemic levels during successive ratoon crops starting from a small amount of infected planting material. In Brazil, losses in biomass of sugarcane due to RSD are estimated to be around 3.3 million tons/yr or R$ 107 million/yr given the price of R$ 32/ton practiced in 2009.

The objectives of our study are a) to establish a time course of colonization of sugarcane by Lxx using the quantitative real time (q)PCR approach in order to identify time points that encompass the onset of the plant reaction to infection; b) to identify sugarcane genes and proteins differentially expressed in a resistant and a susceptible cultivar infected or not with Lxx based on microarray technology at the time points previously defined; c) to characterize the biological effects on sugarcane plantlets of a presumed toxin-like compound secreted by Lxx and study its effects on gene expression in plants cultivated *in vitro*. In addition, genes thought to be involved in the production of this toxin will be characterized by heterologous expression, purification and analysis by mass-spectrometry.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Analyses of gene or protein expression provide a snapshot of an ongoing biological process. Thus, it is crucial to determine when the snapshots will be taken so as to get meaningful answers to the questions being asked. The lack of information regarding the time course of colonization of sugarcane by Lxx prompted us to focus on the establishment of protocols for the inoculation and quantification of Lxx in plant tissue by qPCR in this first semester. With this information, we can define time points of subsequent gene and protein expression analyses. Primers for a Lxx-specific ORF were designed based on its available genome sequence and successfully used in qPCR to quantify Lxx in leaves of young plants. Plants grown *in vitro* (susceptible and resistant varieties) were transplanted to the greenhouse and inoculated after 60 days by cutting them just above the apical meristem and placing a volume of inoculum on the cut surface. Bacterial populations were estimated in leaf DNA extracts 10, 20, 40 and 80 days after inoculation. For optimal quantification of Lxx by qPCR, we found it necessary to extract the plant DNA using a combination of the surfactants SDS and CTAB with proteinase K and lysozyme. The results indicated a rapid in planta growth that sharply contrasts with the slow growing behavior of Lxx in artificial medium. This suggested that a sizeable colonization of sugarcane tissues occurs well before the manifestation of external symptoms, which happen at least 9 months after inoculation. Thus, changes in gene and protein expression in infected sugarcane may occur in a much shorter time span than that expected previously. In addition, we detected differences in bacterial titers between inoculated resistant and susceptible varieties. Further studies should be pursued to determine if this rapid inoculation and quantification method reflects the level of resistance displayed by sugarcane varieties under field conditions. If so, a side result of this study with practical implications would be the use of this technique to select resistant genotypes.

Figure 2. The disease causes significant reduction of biomass in susceptible cultivars of sugarcane. The "stunting" symptom reflects a shortening of the internodes of infected canes (left cane) compared to healthy ones (right cane)

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MATHEMATICAL MODELING OF OPTIMAL BIOLOGICAL PEST CONTROL STRATEGIES FOR EFICIENT AND SUSTAINABLE SUGARCANE PRODUCTION

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Ethanol is a good choice as a fuel and additive because it is produced from renewable resources; promises cleaner combustion leading to a cleaner environment; produces relatively low levels of greenhouse gas emissions over its lifecycle; can be seamlessly integrated into the existing transportation system; provides a new outlet for agricultural products; reduces the global dependence on depleting reserves of crude oil; and has a potential to have a large-scale impact. The increase in world demand for ethanol will bring an increase of the sugarcane planted in Brazil. One of challenges of the improvements in the farming and harvesting of cane is the biological pest control. In spite of the biological control of *Diatraea saccharalis* by *Cotesia flavipes* is considered successful in Brazil , there are some areas where *Cotesia flavipes* has not the good control. The using of the parasitoid *Trichogramma galloi* is considered an interesting option in this case. In other hand, the dynamics of pest – parasitoid populations become complex, making the prediction of outbreaks difficult. Understanding the processes of these interactions can lead to a mathematical modeling playing a decisive role in controlling pest populations and contributing to the stability of natural systems.

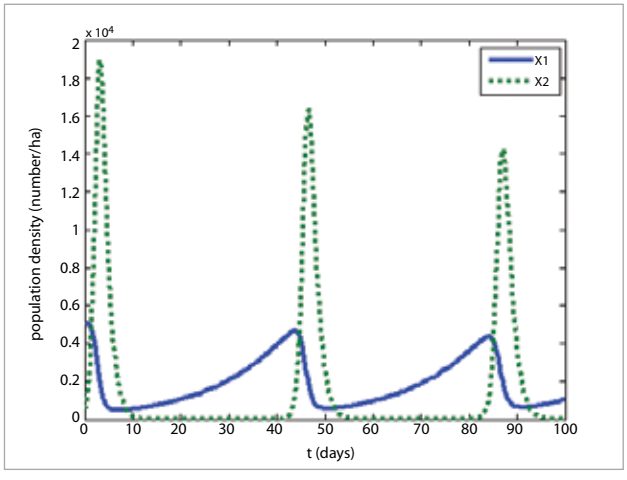

Figure 1. Evolution of sugarcane borer and parasitoid populations without control according to the mathematical model (Rafikov, Angeleli, 2009)

The main aim of this project is to apply methods from optimal control theory and from the theory of dynamic systems to the mathematical modeling of biological pest control strategies.

The specific aims of this project are: modify existing and/or develop new mathematical models adequate to represent interactions between the sugarcane pests and its enemy populations; identify coefficient and parameters of proposed mathematical models and determine the equilibrium level of sugarcane ecosystems from mathematical models; formulate and solve the biological pest control in sugarcane as optimal control problem that determine algorithms of the optimal strategy, minimizing cost functional; elaborate the computational tools based on above mentioned algorithms; undergo numerical simulations for different possible scenarios of biological pest control in sugarcane based on the mathematical models.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The main objective of the biological pest control is to maintain the pest population in an equilibrium state below the economic injury level. Thus, parasitoids and predators are commonly reared in laboratories and periodically liberated in high-density populations (inundative biological control) when the pest population reaches a control level.

For the modeling of first possible scenario, the ecosystem sugarcane borer – its parasitoid was described by two differential equations. For numerical simulations of interactions between the sugarcane borer (*Diatraea saccharalis*) and its parasitoid (*Cotesia flavipes*) were identified the values of model coefficients based on data from literature. *Figure 1* presents the population oscillations without control according to the mathematical model.

The numerical simulations show that the inundative control, applied in initial moment by introduction 20000 parasitoids/ha, maintain the pest population below the economic injury level (2500 pests/ha) only 35 days. After this period, it is necessary to apply the control again.

In order to determine algorithms of the optimal strategy of introduction of the natural enemy species, the biological pest control in sugarcane was formulated and solved as optimal control problem. The linear feedback control is designed to drive the ecosystem sugarcane borer – its parasitoid to the equilibrium state below the economic injury level, as shown in *Figure 2*. Numerical simulations showed that the great amount of parasitoid have to be introduced in initial instance. This fact suggests that the proposed feedback control strategy can be integrated into existing biological control technologies, applying the feedback control after the traditional inundative pest control. This control strategy directs the ecosystem to the stable equilibrium point. It is not necessary to apply the periodic releases or a seasonal introduction of a small population of natural enemies after this control application.

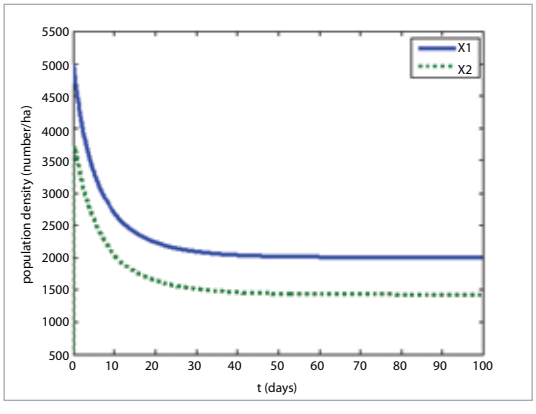

Figure 2. Evolution of sugarcane borer and parasitoid populations with optimal control (Rafikov, Angeleli, 2009)

The next steps of this project will study scenarios which consider age structures of populations and interactions between the sugarcane borer and two parasitoid species.

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ANALYSES OF DROUGHT TOLERANCE IN SUGARCANE USING TRANSCRIPTOMICS AND METABOLOMICS

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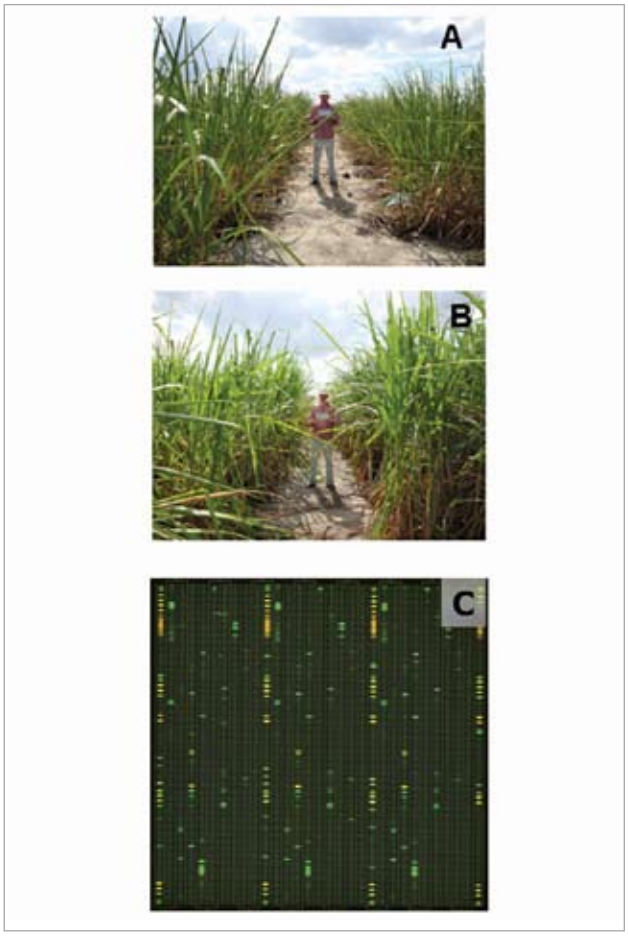

miRNA expression in sugarcane. Plants from RB867515, a drought tolerant variety, were grown for seven months in the field without irrigation (A) or with irrigation (B). RNA was extracted and hybridized with DNA chips (C). Green spots indicate miRNA down-regulated by drought stress, while red spots indicate up-regulated miRNA

We have been working with sugarcane genomics aiming the identification of genes associated to agronomical traits. In this project we aim to identify miRNAs that are modulated by drought stress in sugarcane. To this end we will use genetical genomics, comparing the expression profiles of two groups of sugarcane varieties contrasting for drought tolerance. Each group has two varieties that will be grown in the field, under irrigation or without irrigation. This experiment will capture the plant responses in a real situation of water scarcity. Since field experiments are expected to present high degrees of variation, the same varieties will also be cultivated in greenhouse, in a replicated experiment These experiments will be conducted by Dr. Laurício Endres (Federal University of Alagoas), with whom we cooperate in another project funded by Research and Projects Financing (FINEP) and National Council for Scientific and Technological Development (CNPq). The miRNA expression will be identified using DNA chips containing all known miRNA and also new sugarcane miRNA that will be identified in silico (a second objective of this work). miRNA with interesting expression profiles will be further evaluated by qRT-PCR and *in situ* hybridization. Target genes will be identified in silico and validated by qRT-PCR. The metabolome of same plants used for miRNA analysis will also be evaluated by Dr. Marcelo E. Loureiro (Federal University of Viçosa), who is part of a joint project founded by Minas Gerais State Agency for Research and Development (FAPEMIG). A final objective of this project will be the correlation of the expression profiles of miRNA and their targets with the metabolic changes observed in drought-tolerant and drought-sensitive plants.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

To identify sugarcane miRNA *in silico* we developed a strategy that allowed us to extract 100 new candidate miRNA from the SUCEST database. These new miRNA were printed in a customized miRNA chip containing also all known miRNA, named miRCANA, using the arraying services from LC-Sciences (USA). Sugarcane plants grown in the field presented clear effects of drought stress and differences were observed between drought tolerant and drought sensitive varieties. The hybridization of miRCANA chips was done using RNA from 7 months-old plants grown without or with irrigation. Data evaluation allowed the identification of 12 miRNAs that are associated to drought stress tolerance in sugarcane. The validation of chip data using qRT-PCR from other biological replicates is underway. This will allow us to identify the truly positive miRNA. The experiment with sugarcane plants growing in greenhouse will be finished soon and we expect that it will allow us to obtain data with less variability. It will be interesting to compare the expression profiles in plants grown under field and greenhouse.

MAIN PUBLICATIONS

This is the first year of the project and no publication was produced so far. However, a list of recent publications from our group related to sugarcane genomics is shown.

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MOLECULAR STRATEGIES INVOLVED IN PLANT-INSECT INTERACTIONS

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Figure 1. Sugarcane plants exposed to Diatraea saccharalis *attack and damage produced by the caterpillars after insect wounding and colonization by opportunistic fungi*

Plant responses to insect damage have been investigated and these studies have resulted in new methods to enhance host resistance to insect pests, including the use of insecticidal proteins that can be expressed in selected crops by genetic engineering. Integration of the knowledge of how plants react to insect damage with the techniques of molecular biology should be able to increase even more the methods available for the control of insect pests. Understanding how insects cope with plant defenses also has proved instrumental into designing new strategies for crop protection. The sugarcane transcriptome project (SUCEST) has allowed the identification of several genes involved in the plant response to insect damage. There are numerous classes of naturally occurring phytochemicals that are thought to confer resistance to plants against herbivorous insects. These classes include lectins, waxes, phenolics, sugars, alpha-amylase inhibitors and proteinase inhibitors. Analysis of sugarcane-expressed genes involved in secondary metabolism suggests that most of the expressed compounds may be acting as defensive barriers to insect attack. Our main goal is to understand the defense strategies adopted by sugarcane when challenged by its numerous pests (*Figure 1*). We are also interested in the counter-measures adopted by the insect pests in order to overcome plant defenses. To accomplish these, we are studying leaf proteins and gene responses involved in signal transduction and direct defense of sugarcane plants challenged by *Diatraea saccharalis*, *Spodoptera frugiperda* and *Telchin licus licus*. To understand the insect responses to plant defenses and to evaluate the potential use of genetic engineering to control these insects, we are investigating gene expression and protease activities extracted from intestines of insects exposed to proteinase inhibitors.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

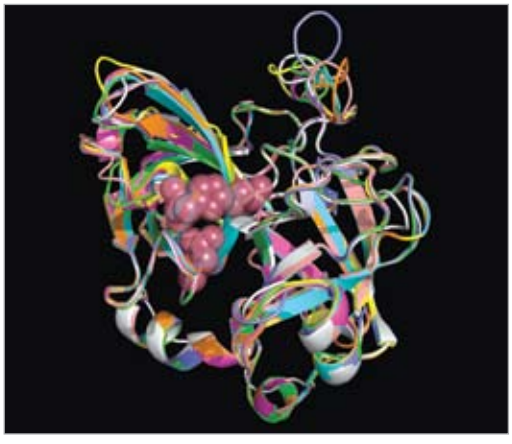

Figure 2. Structural alignment of eight chymotrypsins paralogues modeled by similarity. In detail, the position of the catalytic site

In sugarcane fields, colonization of the stalk by opportunistic fungi usually occurs after the caterpillar *Diatraea saccharalis* attacks sugarcane. Plants respond to insect attack by inducing and accumulating a large set of defense proteins. In a search for defense-related proteins in sugarcane, two homologues of a barley wound-inducible protein (barwin) were identified by in silico analysis, and were designated sugarwin1 and 2 (sugarcane wound-inducible proteins). Using quantitative real-time polymerase chain reaction for monitoring of transcripts, we showed that the induction of sugarwin transcripts is late induced, restricted to the site of damage and occurs in response to mechanical wounding, *D. saccharalis*, and methyl jasmonate treatment. Subcellular localization using GFP indicates that SUGARWINS are secreted proteins. Recombinant SUGARWIN1 protein incorporated into *D. saccharalis* diet, showed no effect on insect development. BARWIN proteins have been described as wound- and pathogen-inducible proteins that possess *in vitro* antipathogenic activities against fungi. We hypothesized that sugarwin gene induction by herbivory is part of a concerted strategy against opportunistic pathogens that are commonly found in the site of caterpillars' attack.

We are also investigating the gene expression profile of proteinases involved in the adaptation when larvae of *Spodoptera frugiperda* are removed from the chronic ingestion of proteinase inhibitors. Larvae of the 6th instar moved to an inhibitor-free diet after the chronic ingestion of PIs showed a decreased in gene expression levels for all proteinases evaluated. Three proteinases showed a distinct pattern of expression when compared with controls: two of them returned to levels of expression below the initial level and one maintained its high level of expression induced by the inhibitors. Our data show that, although transient and dependent of the presence of the inhibitors, the "shotgun" approach changes the initial pattern of proteinases expressed in caterpillars challenged by the inhibitors even after its removal.

Insect proteinases that were induced by challenging the caterpillars with proteinase inhibitors had their tertiary structure modeled and refined in silico by sequence similarity modeling technique. The modeled structures were used to identify possible changes in the structural parameters that might impair the recognition of the catalytic site by inhibitors (*Figure 2*).

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PROCESSING OF SUGARCANE CELLULOSE EMPLOYING ATMOSPHERIC-PRESSURE PLASMAS

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Figure1. Illustration of an atmospheric-pressure plasma jet of Ar/H2 generated by a radio-frequency wave at 50W. In this case, the microplasma jet glow length is about 15.0 mm, considering a gas flux of 700 sccm

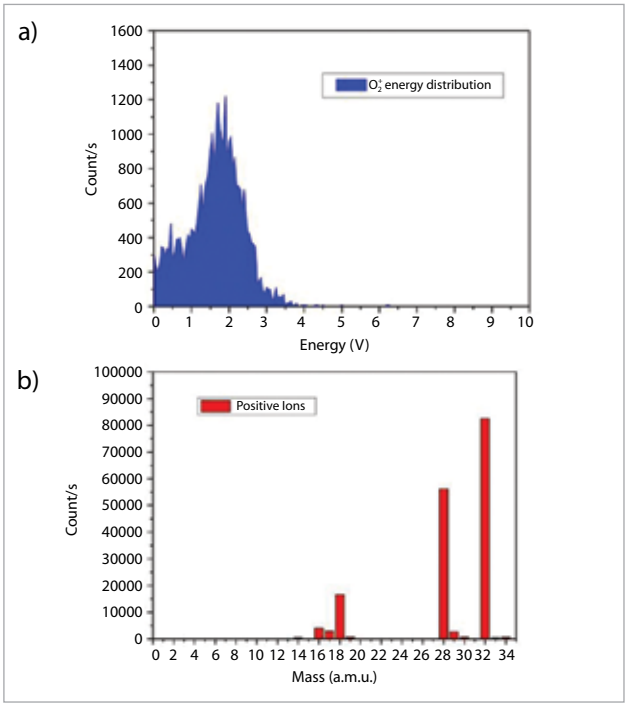

Figure 2. Specimens yielded by He DBD plasma at atmospheric pressure: (a) Energy distribution for O_2^+; (b) Mass concentration of positive ions inside the discharge

In this project we propose the implementation of a new method for selective treatment of lignocellulosic material based on atmospheric-pressure plasmas (*Figure 1*), which may become an important step towards the industrial production of second generation ethanol from sugarcane. The experiments in laboratory scale and the development of the equipment to produce the plasmas will be carried out at Brazilian Bioethanol Science and Technology Laboratory (CTBE). The experiment will be scaled-up in order to be implemented in the Pilot Plant, which is now under construction at CTBE.

From the theoretical point of view, the interaction of the plasma electrons with lignocellulosic material should be better understood. A study on this complex matter will be carried out in three different parts: (1) low-energy electron scattering from α-glucose and β-glucose monomers and dimmers. We expect these results to elucidate the differences in resonant processes responsible for the breakage of the respective (α1→4) and (β1→4) linkages. This study will be carried out at CTBE and the resonance energies should provide invaluable information for optimizing the plasma-based pretreatment of lignocellulosic raw materials. (2) Once the resonance states are identified, we plan to study dissociation mechanisms by electron impact with the help of nuclear dynamics simulations, at Federal University of ABC. (3) Lignocellulosic material contains a large amount of water. We also propose to investigate micro- and macrosolvation effects through some standard approaches coded in quantum chemistry computational packages. This study will be done at University of São Paulo.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

By applying the so-called atmospheric-pressure plasma (APP), low gas and power consumption could be achieved as well as a non-expensive operation [1]. The APP allows creating a convenient environment of chemical specimens, such as ozone and singlet oxygen, which have an important role in the deconstruction of a biomass lignocellulosic matrix, particularly degrading lignin with good efficiency [2, 3, 4, 5]. In this sense, it is necessary to apply diagnostic tools in order to investigate the chemical composition and physical properties of these plasmas. By using Optical Emission Spectroscopy, we are able to determine, for example, the electron density and the temperature of the plasmas [6,7]. Moreover, through Mass Spectrometry Analyzes (MSA), the concentration and energy of neutral specimens as well as of negative and positive ions in the APP can be determined. So far, we have applied MSA to study a helium APP created by a Dielectric Barrier Discharge (DBD). This DBD is going to treat the sugarcane bagasse in a reactor. Some chemical radicals existing in this kind of APP are shown in *Figure 2*.

The first theoretical results are related to the study of elastic collisions of low-energy electrons with the CH_2O-H_2O complex [8]. Previous studies reported a shape resonance for CH_2O at around 1eV. In the presence of water, the resonance appears at lower energies due to mutual polarization between the two molecules. This indicates that the presence of water may favor dissociation by electron impact.

Another interesting theoretical study is the low-energy electron collision with α-D-glucose and β-D-glucose monomers [9]. Our results show a strong isomeric effect for electron impact energies below 15eV. The integral cross sections for both monomers present shape resonances located at different positions. As a consequence, low-energy electrons may dissociate these two monomers at different energies, suggesting a specific bond-selective behavior. The next step is to study electron scattering by the D-glucopyranose dimmers, in order to investigate possible influence of electron capture in the rupture of the ($\alpha 1 \rightarrow 4$) and ($\beta 1 \rightarrow 4$) linkages.

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SUSTAINABLE BIOENERGY SUGARCANE BREEDING AND CULTIVAR DEVELOPMENT

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The sugarcane breeding program of the Agronomic Institute of Campinas (IAC) has developed cultivars adapted for various edaphoclimatic conditions of the Brazilian producing regions. With the changing context for sugarcane production, with an increasing demand for high potential to produce primary energy, a new sugarcane biotype is emerging based on cultivars with increased sugars and total fiber production. Further, the large-scale adoption of mechanical harvesting system in Brazil may promote changes in the pathosystems, with emergence of known and new diseases, a condition that must be considered by breeding programs. Disease resistance is the method of choice to control most pathogens, and it is considered a fundamental aspect in the varietal development, but it has been treated superficially in most breeding programs in Brazil. This proposal suggests a reorientation of the current sugarcane breeding selection processes to attend the new bioenergy demand, by acting in four research topics. The first one is based upon the characterization and selection of clones in advanced stages of experimentation, which adjust to the primary energy potential definition of each selected line. For this, agronomic, phytopathological and molecular characterizations are proposed. The second is applied at the first breeding stages, to utilize a combined selection process to identify and select parents more efficiently for generating progenies with high bioenergy potential. In the third topic, it is proposed to start a genetic introgression program among commercial varieties and *Saccharum spontaneum* genotypes, to promote the incorporation of new genes for sucrose and biomass accumulation, resistance to pests and diseases and adaptation to 'Brazilian cerrado' regions ('drought-prone environments'). The last one aims to investigate the existing genetic diversity of the main sugarcane pathogens in the distinct Brazilian producing regions, to enable a more efficient selection of resistant/tolerant genotypes. The results should contribute with sustainable sugarcane cultivation, while contributing to keep the Brazilian leadership in sugar and biofuel production.

Figure 1. Photoperiod facility for flowering induction and hybridization

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Figure 2. Nucleus of sugarcane seedling production

- Establish a collection of parents with high potential to generate families with high performance on sucrose and biomass production;
- Promote germplasm enrichment in Brazil, through the introduction of new materials;
- Identify and characterize new sources of germplasm;
- Promote the broadening of the genetic base of IAC varieties;
- Establish procedures for synchronization of flowering under controlled conditions, allowing interspecific crosses under Brazilian condition;
- Establish methods to diagnosis genotype responses to the main pathogens of sugarcane;
- Recognize pathogen diversity and population structure in main producing regions to help breeding for disease resistance;
- Identify genotypes resistant to pests, nematodes and diseases, target of this project;
- Disseminate the results obtained on national and international meetings and on journal of selective editorial policy.

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NATIONAL INSTITUTE OF SCIENCE AND TECHNOLOGY OF BIOETHANOL

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In the 70s, Brazil started a program to substitute gasoline by ethanol in order to decrease dependence from politically and economically variable periods. The plant species chosen was sugarcane and as a consequence, agricultural and technological studies were greatly intensified, leading Brazil to a very favorable position in terms of energy security. Nowadays, Brazil has more than 80% of its cars running with bioethanol and even airplane engines are now being developed. With the increasing political instability in the Middle East, since 2001, the USA has also decided to direct its energy policy towards the use of biofuels. This is now being followed by Europe and Japan and it is likely to be followed by several other countries in the world. This imposes an enormous pressure on the production of crops that can supply bioethanol.

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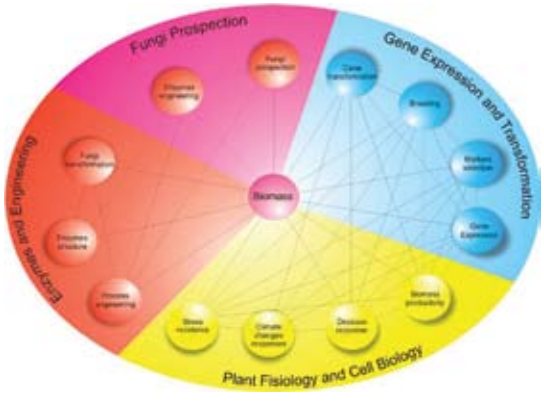

Figure 1. Structure of the INCT do Bioetanol, which is divided in 4 centers that congregate 30 laboratories in 6 states of Brazil

Brazilian sugarcane is probably the most efficient extant bioethanol producing system. However, only part of the biomass produced is used for bioenergy production, 1/3 of the plant being used for sucrose production, 1/3 is bagasse, which is burnt for production of electricity and the last third is left in the field and latter on decomposed by microorganisms. Therefore, in order to supply wider needs, a significant increase in production of ethanol is possible if we can provide the basic knowledge necessary for development of technologies that will be capable to obtain energy from the polysaccharides of the cell wall, which makes 70% of the biomass burnt inefficiently and left in the field. The availability of such processes within the distillery and the higher marketing value of liquid fuel provide additional economical advantages to its conversion instead of simply burning bagasse. Although the chemical hydrolysis of biomass is a consolidated methodology under laboratory conditions, its large-scale application is not economically viable, yet. The necessary use of acids reduces the life-time of equipments, produces toxic wastes and produces non-fermentable sugars, increasing the costs of the products. One alternative is the enzymatic hydrolysis of the cell wall. Such a process requires the use of a complex machinery of specific enzymes that are produced either by the plant itself or by microorganism able to degrade plant cell walls. On the other hand, relatively little is known about the structure and architecture of the cell wall. One of the goals of the INCT do Bioetanol (*Figure 1*) is understand the fine structure of the principal hemicelluloses of sugarcane and other possible sources of biomass. We intend to find patterns of gene expression that could be useful to find ways to induce the plants to degrade their own wall and become prepared for subsequent hydrolysis. In parallel, we intend to prospect microorganisms, enzymes and genes both in microorganisms and sugarcane, that are capable to efficiently hydrolyze the walls. Such enzymes will be designed to have the highest possible performance to degrade plant cell walls, especially the walls of sugarcane. A group of researchers screen existent varieties of sugarcane to find gene markers that could guide the groups to quickly identify plant materials that would be more suitable for use in industrial processes. From the latter viewpoint, our group intends to perform tests of mechanical preparation of sugarcane for further acid and/or enzymatic hydrolysis. With this data in hands, we expect to be able to provide the fundamental knowledge of biotechnology that is necessary for scaling up studies and further increase in efficient of bioethanol production in Brazil.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

UNDERSTANDING HOW SUGARCANE PLANTS FUNCTION

1) Sugarcane has been deeply studied regarding its physiological traits so that the latter could be related with the genetic markers under development by breeders and help to find superior sugarcane varieties in many senses. To do that, plant physiologists started to construct a databank that will be available to other researches;

2) To understand how sugarcane will respond to the global climatic changes, plants have been grown in elevated CO2 and it was found that its photosynthesis and biomass increased considerably. Researchers discovered that plants capture more light to compensate elevated CO_2 and activate genes related to the electron transport system. Now researchers will try to increase gene expression of photosynthesis to see whether growth is positively affected in the same way, but without the extra CO_2;

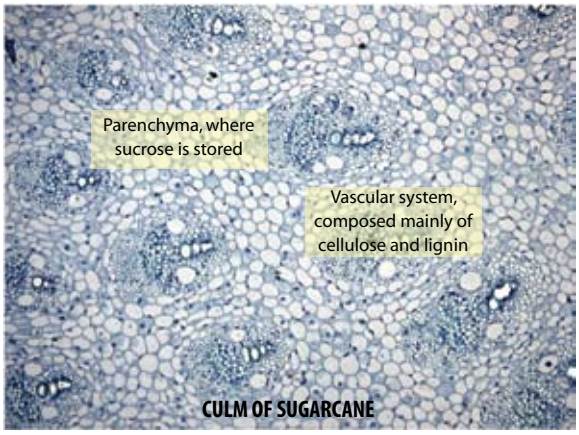

Figure 2. Section of the culm ("stem") of sugarcane showing where sucrose and walls are in the tissue. This picture shows the general structure of what has to be degraded by enzymes to produce free sugars for fermentation and production of bioethanol

3) With the development of molecular markers, it has been possible to map the genome so that researchers are starting to find genes that can indicate important features related to the agronomic features of sugarcane, such as higher productivity, resistance to drought, higher sugar and fiber contents;

4) The expression of important genes and proteins, related to photosynthesis, drought resistance, sugar content and cell wall metabolism are being studied. This information, together with the physiological data, will be important to design strategies to understand how sugarcane plants function. This information can be of great help to breeders as they could produce varieties (or modify plants genetically) that will be more productive and better adapted to different environmental conditions throughout the country.

PRODUCING THE BASIC SCIENCE FOR THE CELLULOSIC ETHANOL

1) The sugarcane cell wall had its chemical structure determined and the polysaccharides have been subjected to hydrolysis with fungal enzymes to understand their mode of action;

2) Sugarcane tissues were sliced and analyzed anatomically (*Figure 2*). We have now enough data and are producing an Atlas that will permit researchers to understand plant better structure of the tissues that have to be degraded to produce bioethanol;

3) Sugarcane bagasse has been characterized and pre-treatment systems are under intensive focus, especially the use of acid hydrolysis and steam explosion;

4) The chemical structure of the trash left in the field has been followed for over a year and the quality of this material, important for use as raw material for second generation bioethanol, is now established;

5) Several fungi species have been found to produce enzyme cocktails capable to degrade sugarcane cell walls. Many enzymes of these cocktails have been purified, their genes cloned and heterologously expressed in bacteria;

6) Yeast species were found that are capable to metabolize pentoses such as xylose and arabinose.

7) Enzymes have been crystallized and some were engineered by artificially introducing catalytic sites from laccase and xylanase in the same protein. These enzymes are being tested with different substrates.

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SUGARCANE GENOME SEQUENCE: PLANT TRANSPOSABLE ELEMENTS ARE ACTIVE CONTRIBUTORS TO GENE STRUCTURE VARIATION, REGULATION AND FUNCTION

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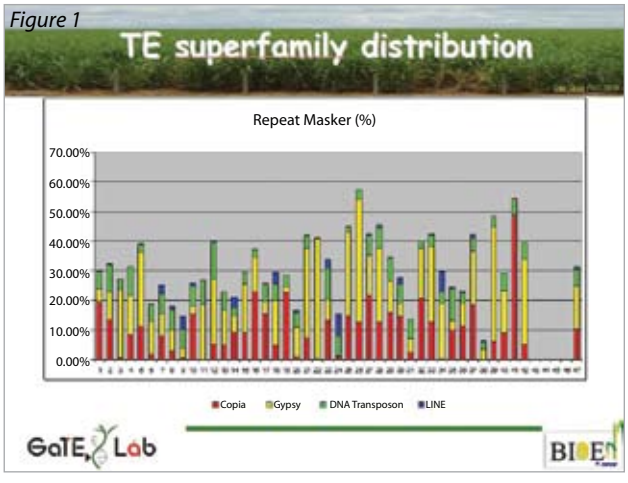


Figure 1

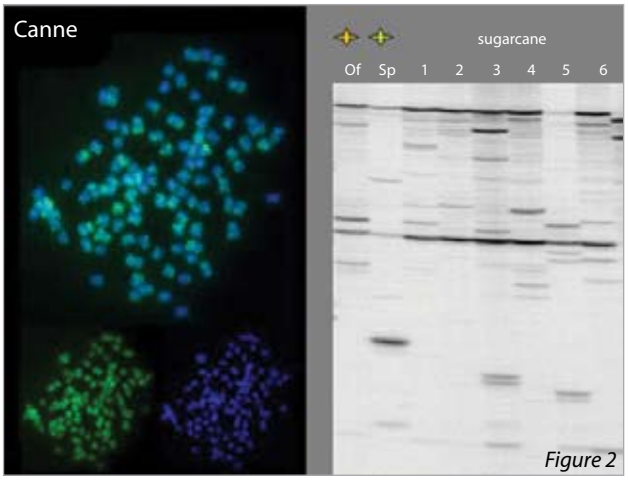


Figure 2

Sugarcane is the major feedstock used in Brazil for biofuel production. It corresponds to one of the largest commodities of the agribusiness in the State of São Paulo. Bioethanol production is dependent on sucrose as the major starting material. Brazilian Sugarcane Industry competitiveness is expected pending the increase in total yield and avoidance of the use of new land for farming. Increase in biomass production is expected through modulation of sucrose metabolism under beneficial and restrictive growth environments, such as drought. In addition, efficient utilization of bagasse as biomass is mandatory to the whole chain production net yield. This project aims at generating a draft sequence from two specific sugarcane cultivars (R570 and SP80-3280) so that tools are generated for understanding genome polyploidy variation, enable gene discovery and generate a knowledge base molecular infrastructure. Basic research will benefit not only from gene discovery but from the identification of regulatory sequences involved in sucrose metabolism, carbon partitioning in the plant and responses to restrictive water supply. Breeding programs will have access to the development of new molecular markers. Sugarcane corresponds to a highly polyploid genome among grasses. It is a recent hybrid generated by crosses between *Saccharum officinarum* and *Saccharum spontaneum* and its monoploid genome is estimated to be about 1Gb comparable in scale to the human and maize genomes. It is proposed to tackle the sugarcane genome by a combined approach of 454 pyrosequencing and Sanger sequencing of 1000 BACs. Available resources are an EST collection generated by SUCEST, array hybridization profiles generated from SUCEST-FUN and a collection R570 BAC clones. BIOEN program will generate a SP80-3280 BAC library which will be screened for homologous R570 BAC sequenced locus to address allelic variation not only in coding regions but also on regulatory sequences. Transposable elements (TEs) mapping onto these sequenced BACs, array based expression profiles and insertion polymorphism study will provide information concerning their association to genetic diversity in sugarcane crop design. The ultimate goal is to contribute with a large scientific community effort to improve sugarcane breeding and develop a systems biology based approach in sugarcane plant biology.

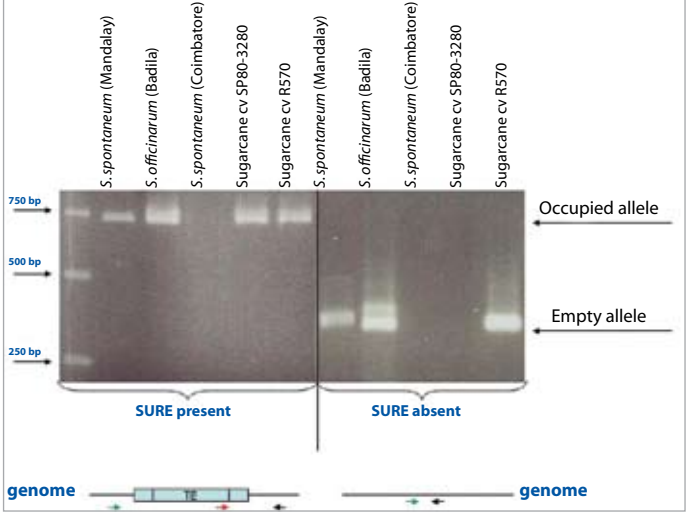
SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Over 50 sugarcane BAC clones have been sequenced and 42 of these have been thoroughly examined for their TE content. Classification of the elements was made using broad lineage classification as DNA transposons, Ty1/Copia and Ty3/Gypsy, both LTR-retrotransposons and LINE retroelements as depicted in *Figure 1*. These BAC clones were selected for different set of genes and display no particular TE enrichment except for a larger proportion of LTR-retrotransposons. Preliminary results suggest a negative correlation of Ty3/Gypsy elements with gene rich regions.

scALE LTR retrotransposons belong to Ty1/Copia lineage and were the most abundant transcript in SUCEST. In this particular case, copy number correltates with higher expression level suggesting that this element is potentially active. Also, BAC analysis correlates its presence with gene rich regions. FISH hybridization pattern presented in *Figure 2* supports random distribution of the lineage in sugarcane chromosomes. Recent transposition activity is supported for another LTR-retrotransposon based on insertional profiling also presented in *Figure 2*.

The insertion pattern of these LTR-retrotransposons can be converted to molecular makers to be used in breeding programs as these elements usually do not excise from their insertion locus. *Figure 3* present the analysis of the insertion of a third Ty1/Copia lineage named SURE (SUgarcane REtrotransposon 1) in a particular locus from BAC 11K15. Interesting to note is that some genotypes possesses unique patterns such as *Saccharum spontaneum* cv. Coimbatore, and others have both alleles with and without the occupied locus supporting the polyploidy genome richness and complexity. Moreover, the results presented suggest that this particular insertion shared by both *Saccharum spontaneum* and *Saccharum officinarum* genomes.

Figure 3



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ENERGETIC HOMEOSTASIS AND SUGAR SIGNALING: DIVERSIFICATION OF THEMOLECULAR MECHANISMS INVOLVED IN THE CONTROL OF THE ENERGETIC BALANCE IN ANGIOSPERMS

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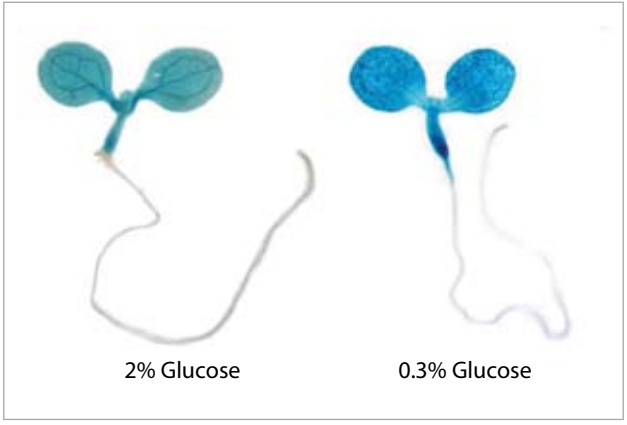
To optimize their growth and development, plants, as sessile organisms, have developed a range of efficient mechanisms to sense and respond adequately to ever changing environmental conditions. The production of sugar through photosynthesis primarily relies on light accessibility.

These photosynthetic-derived sugars represent important signals, which, in combination with developmental and environmental cues, such as mineral nutrition, water availability or pathogens attacks, influence the use of energy resources to ensure survival and propagation. Interaction between developmental, hormonal and sugar regulatory signals is deeply involved in growth control and ultimately in biomass production. The molecular mechanisms responsible for the cross talk between these different signaling pathways and their diversification in plants still need to be further elucidated to better understand plant growth patterns and biomass production. Overall, the present proposal aims at unraveling new mechanistic aspects of sugar signal transduction in plants. More specifically, we intend to: 1) define the diversification of glucose and sucrose-induced gene expression programs among angiosperms (sugarcane, rice and Arabidopsis); 2) evaluate and describe glucose-mediated mRNA stability; 3) characterize the function of bZIP transcription factors mediating glucose-related processes; 4) provide new insight into mannose signaling. We anticipate that the data will improve our view of sugar signaling and energy homeostasis control in plants and the results will be integrated into databases that could feed projects related to biomass and bioenergy research.



Transgenic Arabidopsis thaliana *plants expressing* AtbZIP63 *promoter:* gusA *fusion was generated and GUS expression in seedlings grown in 2% and 0.3% glucose show differential levels of GUS activity*

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INTERNATIONAL BIOENERGY MARKET: ASSESSING INSTITUTIONAL STRUCTURES

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Figure 1. Illustration of a multilateral market: Commodity Exchange in Brazil (BM&F)

The fast-growing global bioenergy market, and the particular features of its demand bring into question the production and trade of renewable products and how they should be regulated in the years to come. Institutional structures governing renewable energy trade are costly and still vaguely defined. That is the most likely reason why the majority of international trade of bioenergy products (mainly ethanol) is based on bilateral and idiosyncratic long-term contracts instead of multilateral transactions. Different from regular commodities markets, the emergence of market institutions in the international trade of bioenergy products requires mechanisms that transmit information about socio-environmental sustainability, inasmuch as this is an essential feature of its growing demand.

We propose to analyze the institutions for the emerging global market of bioenergy products, in particular the capability of those institutions to encompass the production of a broader range of third world countries, so as to increase the number of suppliers and to mitigate risks associated to regional supply shocks, such as draughts or political instability. A more reliable supply of bioenergy products seems a necessary condition for fostering the international demand by energy consumers, particularly for the adoption of mandates and other mandatory consumer measures.

This task is complex because bioenergy products are by large credence goods, i.e., they must carry the information of environment sustainability, which drives the demand for bioenergy products. As a consequence, specific coordination is required in order to establish international standards, and to build institutional arrangements to transact information about the credence aspects of those products, both oriented to the reduction of transaction costs in this emerging market.

This research work aims to understand how institutions of international bioenergy markets will develop in the next years, and what the most relevant impacts will be on the competitiveness and production sustainability in Brazil and other potential producers, particularly in Latin America and África. By understanding better the factors involved in the development of bioenergy market institutions, we expect to support public policies oriented to the sustainable development of third world countries, as well as to stabilize the geopolitics tensions derived from the regional concentration of oil-based energy.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

This research began in late 2009 with the following expected results.

We first intend to map the current governance structures observed in bioenergy international transactions. Preliminary evidence suggests that those transactions are mainly governed by means of idiosyncratic bilateral contracts, with several monitoring devices in order to guarantee that bioenergy products meet socio-environmental required standards. There are also several certification mechanisms now that are likely candidates for assuring the social-environmental features of bioenergy products. The competition among certification mechanisms is based on network externalities and interest groups coordination failures, which make it possible to foresee the ones that will prevail in the near future.

The next step is to measure the transaction costs of current governance structures, and those of the multilateral exchange based on certifications, by means of two distinct methodologies (World Bank's and Ronald Coase Institute's). At this stage of the research, it will be possible to predict if the international bioenergy trade will move to a multilateral market, if this path will benefit from specific public policies, and what will the expected gains be from this new institutional arrangement. It is noteworthy that the available methodologies for estimating transaction costs actually focus on observed costs, providing just a partial (although important) picture of governance structure efficiency. The need to take a broader perspective on the dynamics of the competing institutional mechanisms takes this research to the third set of expected results.

The development of multilateral market institutions based on certification mechanisms will depend not only on the features of current governance structures, and on the availability of inputs for bioenergy production. The conversion of other countries into bioenergy suppliers – a relevant variable to increase supply reliability and, as a consequence, to foster the international demand for bioenergy products – depends also on the institutional environment in those countries. This research will confront the social-environmental standards demanded by international consumers and the institutional structures of third world countries – mainly from Africa and Central America – in order to access the likelihood of the dissemination of the Brazilian successful experience and the constitution of a multilateral bioenergy market.

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CONTROL OF LIGNIN BIOSYNTHESIS IN SUGARCANE: MANY GAPS STILL TO BE FILLED

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Lignin content may vary in response to several biotic and abiotic stresses and understanding how this occurs may help to understand how to control the lignin biosynthesis and consequently its content in plants. Very little is known about lignin metabolism in sugarcane. However, taking in account the information accumulated for other plants and the agronomical practices and problems in sugarcane cultivation, we may have enough hints to plan several studies on how sugarcane modulates lignin composition and content. Therefore, the aim of this project is 1) to cultivate contrasting sugarcane genetic material for lignin content in six locations well characterized for temperature, water availability and irradiance and analyze lignin, sucrose and cellulose, and then, based on these results to study gene expression and perform a more detailed study of lignin composition; 2) to search the SUCEST database for ESTs coding transcription factors known to be involved in lignin metabolism in model plants and use this information in controlled studies (on water supply, nitrogen fertilization, light intensity and low temperatures under field and greenhouse conditions, and growth chamber) to establish correlations between transcription factors regulation and lignin content and composition; 3) search the SUCEST database for ESTs coding ortologs to peroxidases and laccases and use this information in the controlled studies to evaluate the involvement of these enzymes in lignin biosynthesis; 4) to perform a system biology study of regulatory network involved in lignin biosynthesis. To reach these aims we will use a population segregating for lignin content. With this information we may get some valuable knowledge on the lignin biosynthesis in the complex sugarcane genome. Shortly we believe to have at least initial information on how environmental factors affect different sugarcane genotypes. Also it is expected that our results allow us to understand how genes of the lignin biosynthesis pathway and related transcription factors changes lignin content and structure in sugarcane as influenced by some specific environmental factors. In a long-term objective, we may have the possibility to gain knowledge on the agronomical practices may contribute to decrease lignin in sugarcane. This knowledge on the interaction of environmental factors and control of the lignin biosynthesis and related transcription factors may allows us to design a transgenic plants altered in specific genes, aiming not only the decrease of lignin but also its structure.

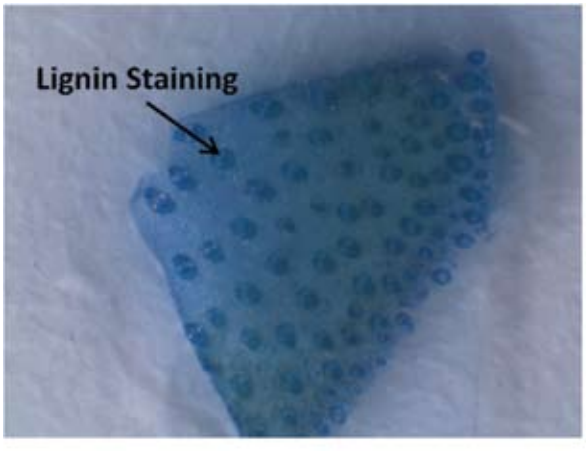

Figure 1. Lignin Staining

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

1) selected the sugarcane material to be planted in the six experimental fields;

2) installed greenhouse experiments for controlled studies (on water supply and nitrogen fertilization);

3) started the analysis of the SUCEST database crossing sequence and functional information with the rice, sorghum and Arabidopsis genomes in order to select sequences for lignin biosynthesis including for peroxidase and laccase and transcription factors;

4) started to map the lignin distribution in the sugarcane plant in order to understand the temporal deposition and distribution in the cane.

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CONCENTRATED VINASSE USE ON SUGARCANE PLANTS: SOIL CHEMICAL ATTRIBUTES, ION LIXIVIATION AND AGRONOMIC EFFICIENCY MONITORING

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Figure 1. Vinasse application in the plot experiment

The increasing ethanol production represents many advantages to Brazil as a less pollutant renewable fuel, but it will generate higher quantities of vinasse, the residue that is applied as a fertilizer for sugarcane. On the other hand, it is already known that there is a potential risk of ion lixiviation to the underground waters when high rates of vinasse are applied to the same soil during several years. In this way, other uses for vinasse shall be studied, but the high vinasse value as an organic fertilizer turns indispensable to find out new viable procedures for its use on sugarcane cropping. In this sense, the concentrated vinasse might be one alternative to take the residue to more distant soils, contributing to the sugarcane nutrition with significant savings for the country on imported fertilizers. However, further investigation is urgently required to find out whether the concentrated vinasse is a potentially higher pollutant residue than the natural vinasse. In order to evaluate concentrated vinasse effects on soils and its potential risk of ion lixiviation to underground waters, some trials are being carried out. The specific aims are: (1) Compare the normal and concentrated vinasse physical-chemical characteristics from several samplings from the same factory, and also, to compare their characteristics with the parameters established by the legislation for agriculture use. Besides, to evaluate the concentrated vinasse residue effects on soils, in order to obtain knowledge to its sustainable use in crop ferti-irrigation mainly to sugarcane cropping; (2) Evaluate the concentrated vinasse biodegradation and mineralization compared to the normal vinasse; (3) Evaluate the ion lixiviation potential of concentrated vinasse compared to the normal vinasse and to the mineral potassium fertilization; (4) Evaluate the concentrated vinasse agronomic potential as a source of nutrients to sugarcane crop.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

This project started in November 2009 by plotting the field experiment. The area used was located in Batatais region, São Paulo State, and the sugarcane was in the first ratton stage. The results reported at the present are still preliminary being the conclusions expected in September – October of 2010 by harvesting the plants and analyzing all the samples for monitoring vinasse uses.

In the experiment, samples of two kinds of vinasse were applied, one is the vinasse that is normally obtained in the ethanol production (normal vinhasse), and the second is a concentrated vinasse, both proceeding of the same sugarcane mill. A scheme for sampling and analyzing vinasse samples periodically was established so the different vinasses were compared (*Table 1*). The results showed that chemicals properties of both vinasses are similar, however macro-nutrients concentrations, especially potassium (K), are higher in concentrated vinasse. There were variations on macro-nutrients contents in vinasse through the months, but in general, K concentrations have been 20 to 30 times higher in concentrated vinasse.

Soil samples, obtained from the field experiment, were taken to the laboratory to evaluate carbon (C) and nitrogen (N) mineralization after normal vinasse and concentrated vinasse application. The preliminary results of these tests showed that C and N mineralization occurs faster with normal vinasse when compared to concentrated vinasse.

Table 1. Macronutrients and pH in concentrated vinasse compared with normal vinasse

	pH	N	PO_4I	K_2O	Ca	Mg	SO_4
				gL^{-1}			
Vinasse	4,1	0,35	0,08	2,18	0,19	0,35	1,1
Concentrated vinasse	4,1	3,7	1,3	52,8	3,1	4,4	18,2

N – Kjeldahl, K – flame photometry, P, Ca, Mg, SO4 – ICP-AES

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ASSOCIATION ANALYSIS USING SSR AND SNP LOCI TO FIND QTL FOR SEED OIL CONTENT IN SOYBEAN

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Figure 1. Different soybean accessions under the same field conditions

Brazil is known by its great variability and seed yield that may be used to extract vegetable oil or biofuel. Nowadays, soybean is the best alternative among all oilseed crops, mainly because of the huge cultivated area throughout the country, the adequate levels of productivity and quality of oil (viscosity and cetane ratio), the short-term cycle (nearly four months from seeding to harvesting) and the network of crushing industries available nationwide.

The objective of this research is to determine the positions of Quantitative Trait Loci (QTL) and the level of polymorphism of candidate genes associated with oil production and fatty acid composition in soybean populations.

The mapping population of 96 accessions, includes Brazilian modern cultivars, Brazilian lines and Plant Introductions from USDA (United States Department of Agriculture) germplasm collection. This population was divided into two subpopulations of 48 accessions each: one with high (more than 22%) and other with low (less than 17%) oil content. Recently, they were planted in the experimental area of ESALQ/USP.

The accessions will be analyzed with 100 SSR (Simple Sequence Repeat) loci that were selected based on their association with QTL for oil content or polymorphism shown in previous studies. Concurrently, we will sequence portions of up to 10 candidate loci involved in oil biosynthesis in a sample of 10 soybean accessions selected to high and low oil. Single Nucleotide Polymorphisms (SNP) identified in the sequences will be used as markers for those candidate genes. SNPs that cause phenotypic variation are priority for genotyping. Collaborators will collect data on the level of fatty acids in each of the accession. Standardized Disequilibrium Coefficients (D') and Squared Allele-frequency Correlations (r2) for pairs of SSR and SNP loci will be used to estimate Linkage Disequilibrium (LD). The seeds' oil content will be tested for significant differences in allele frequencies between the low and high oil groups. Putative QTL may be identified on the basis of highly significant markers, which will certainly become a useful tool in future breeding program to rapidly increase the rates of oil content.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

In a total of 128 SSR loci tested, 100 were polymorphic in a sample of 10 different soybean accessions (*Figure 2*). To each SSR loci the best reaction conditions were optimized in order to reach the better PCR product. Amplicons and marker data of those preliminary tests were detected using a 4300 DNA analyzer and Saga GT software from LI-COR Corporate. The following steps include the determination of allelic frequencies, allele size, and genetic diversity in the mapping population.

In Brazilian field conditions, the 96 soybean accessions have shown high variability in days of flowering, plant maturity, disease resistance, and grain yield.

Preliminary results of the same accessions developed in greenhouse conditions of seed oil content by Ressonance Magnetic Nuclear (RMN) also showed high level of variability (from 11% to up to 25%)(*Figure 3*).

The presence of high genetic variability among soybean cultivars for oil content and seed yield can help to determine regions in LD with SSR and SNP loci. The results suggest that the SSR loci, together with a high standard of variability of the plants will provide a consistent analysis of soybean germplasm.

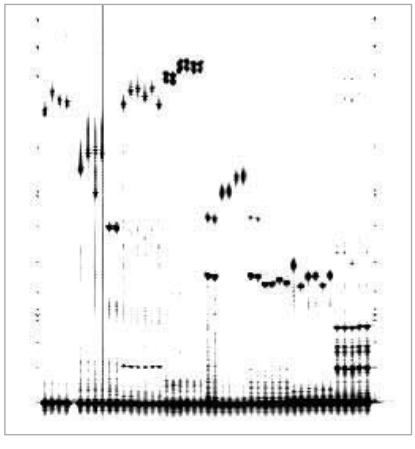

Figure 2. Polymorphism of eight SSR loci showed by electrophoresis in 4300 DNA analyzer

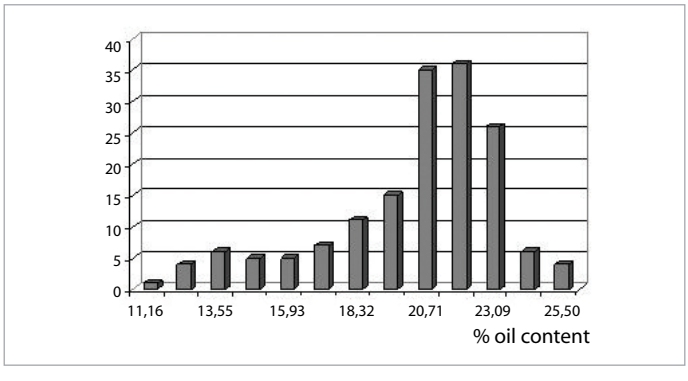

Figure 3. Distribution of oil content (%dry mass) in 96 soybean accession development in Greenhouse conditions

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SUGARCANE ENERGETIC BALANCE: A SYSTEMS APPROACH TOWARDS UNDERSTANDING REGULATION OF SUCROSE METABOLISM AND SUGAR SIGNALING

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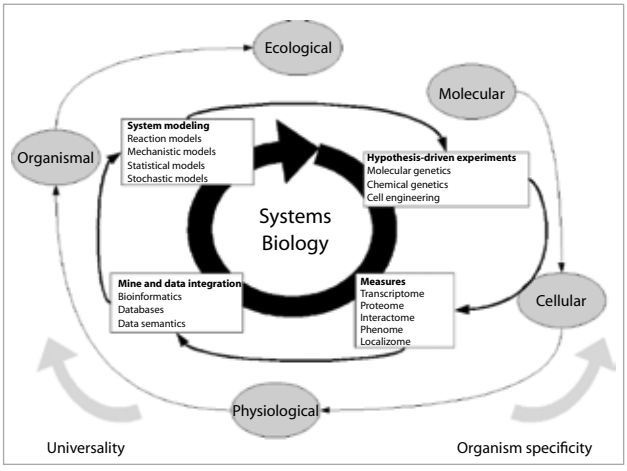

Figure 1. Systems biology overview

Our laboratory has dedicated in developing Systems Biology approach for understanding molecular and physiological aspect of plants species. Sugarcane research has identified and characterized a suite of proteins involved in carbon biosynthesis and sugar sensing. However, current results towards understanding sucrose biosynthesis and accumulation have fallen short of expectations. The molecular mechanisms responsible for the cross talk between these different regulatory and signaling pathways and their diversification in plants still need to be further elucidated to better understand plant growth patterns and biomass production. We are only beginning to produce the detailed gene expression data needed for understanding the network of interactions at a molecular level. To address the rate of gene discovery, high-throughput approaches have being developed for biological experimentation and relevant biological questions regarding gene, protein interactions or networks of biological process can now be addressed. Here, we propose to develop a research approach which integrates molecular and systems biology to improve the knowledge about carbohydrates biosynthesis and sugar regulatory signaling in sugarcane. In this research project we will elaborate ways to apply analyses of regulatory network and dynamic metabolic models in molecular and genetic data of sugarcane related with sucrose biosynthesis, and define the diversification of glucose and sucrose-induced gene expression programs among angiosperms. We expected that the models captures the regulation of many sugarcane genetic components and anticipate that the data will improve our view of sugar signaling in plants. Simulations of our models will provide an efficient tool for the identification of candidate to genetic manipulations that have the best chance to promote increase in sucrose content and for the prioritization of future analyzes.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

A long-term goal of sugarcane growth modeling is to be able to do analysis based on gene expression data organized into genetic and metabolic pathways that are modeled for interactions. As an initial step towards an evaluation of the evolutionary conservation of sugar signaling in angiosperms, we are analyzing the short term (0h, 15 min, 2h and 4h) regulation of sugarcane, sorghum, rice and Arabidopsis signal transduction components by glucose, fructose, sucrose, trehalose and 3-oxy-methyl-D-glucose. In previous study, 58 genes were regulated by glucose including 44 up-regulated and 14 repressed genes. On the other hand, sucrose treatment resulted in 55 differentially regulated genes, 46 of which were induced while 9 were repressed as compared to the untreated sample. A sugarcane cDNA microarray previously described was used to assess the gene expression program of mature leaves. Three ESTs coding for 14-3-3 proteins were found to be more expressed in mature leaves from a low sugar content population. It was suggested that the members of this family affect carbohydrate metabolism by binding to SPS. This enzyme has several putative phosphorylation sites that regulate its activity by 14-3-3-dependent and -independent mechanisms. Phosphorylation by a kinase, such as SNF1, does not inactivate SPS, but tags the enzyme for 14-3-3 binding which completes the signal-induced transition towards inactivation. In line with these data, the up regulation of three ESTs coding for 14-3-3 proteins in low sugar mature leaves could reflect the inactivation state of SPS and consequently the low sugar content in these plants. Our finding may indicate that the decrease in the expression of these genes in the mature internodes may allow for sucrose accumulation. The knowledge that will be produced by this research will be useful to refine the current acknowledgment and to develop a global model of sucrose metabolism and allocation. Clarification of how the sink acts to regulate source activity in sugarcane will provide researchers with additional potential targets for manipulation towards improving sucrose yield.

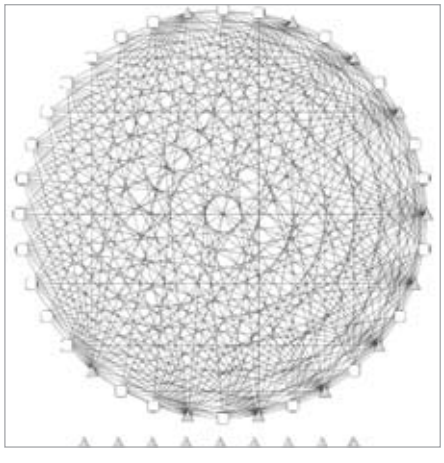

Figure 2. Regulatory network representation of genes coexpressed in sucrose biosynthesis process

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BIOPROCESS SYSTEMS ENGINEERING (BSE) APPLIED TO THE PRODUCTION OF BIOETHANOL FROM SUGARCANE BAGASSE

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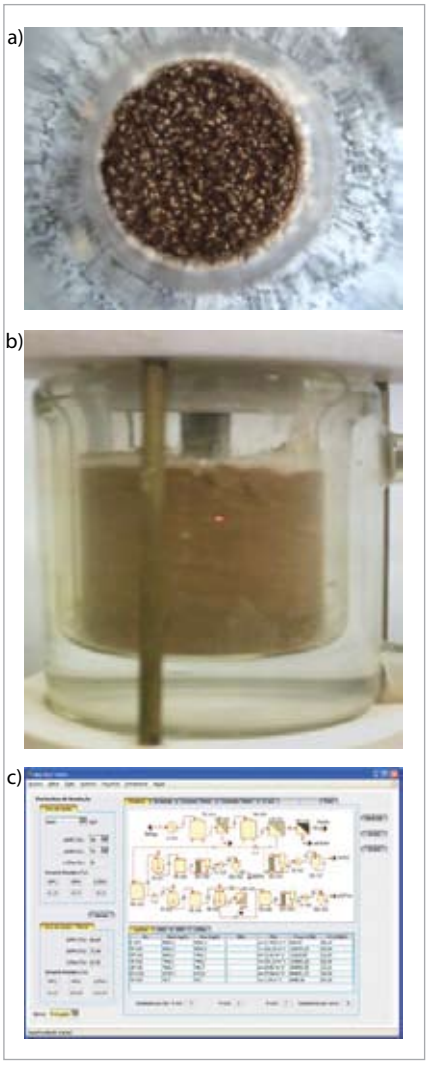

Figure1. From micro scale to plant-wide optimization: (a) Cellulase-production fungus on bagasse substrate. (b) Lab scale bioreactor operating with immobilized pool of cellulolitic enzymes. (c) Biorefinery flowsheet: graphical user interface of the simulation toolbox of the web application

The industrial production of biofuels, understood as fuels produced from biological feedstock, is presently at what can be called a technological crossroad, and a hard competition among different technologies is in course. The winners will be defined by a combination of economical criteria, process robustness and compliance to environmental and sustainability restrictions. In this scenario the optimization of this complex, interconnected process ideally must be pursued ever since its early stages of development, aiming at costs reduction, negative overall CO_2 balance, cutback of water usage and of effluent emissions and so forth. Fine-tuned processes, operating at (near-) optimum conditions will have a significant competitive advantage.

This project focuses on the rational application of (Bio-)Process Systems Engineering (BSE) techniques to the process for production of bioethanol from an important lignocellulosic material in the Brazilian scenario, sugarcane bagasse. In other words, the same approach that allowed oil refineries to achieve a high productivity is herein applied to biorefineries.

The validation of BSE tools for assessing different routes for bioethanol, however, must be based on real data. With this purpose, this project joins efforts of a group of researchers from the Chemical Engineering Department of UFSCar and from Brazilian Agricultural Research Corporation (EMBRAPA Agriculture Instrumentation). A biochemical route for production of ethanol from sugarcane bagasse is our selected case study, encompassing different technologies, some of them still exploratory: *in situ* production of cellulases in triphasic reactors; feedstock pre-treatment; enzymatic production of pentoses (and their transformation) and of hexoses via a non-conventional process using immobilized enzymes, combined with simultaneous (SSF) or consecutive fermentation (CSF), using *S. cerevisiae* in conventional and non-conventional bioreactors. A global view is necessary to integrate these processes from the early stage of development. Therefore, this project aims simultaneously at providing the necessary software and at researching new feasible routes for bioethanol which, in addition to their intrinsic value, will be employed for validation of the methodology.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

This project, presently in its first year, will implement the necessary software while researching new feasible routes for bioethanol which, in addition to their intrinsic value, will be the case study for validation of the methodology. It is important to notice that the proposed computational applicative, within a web-based environment, may serve as a support tool for other projects within the BIOEN program. It includes the Laboratory for Development and Automation of Bioprocesses (LaDABio), the Laboratory of Enzymatic Process Engineering (LabEnz) and the Biochemical Engineering Group, all from the Department of Chemical Engineering of the Federal University of São Carlos (DEQ/UFSCar) and the Bioprocess Group of the EMBRAPA Agriculture Instrumentation unit, in São Carlos. Cooperation other groups is also evolving (including PEQ/COPPE/UFRJ and DEQ/UFRGS). The project research lines are:

- Development, implementation and validation of a user-friendly integrated computational environment, enabling simulation, optimization, economic evaluation, CO_2 and water usage assessment, analysis of kinetic data.
- Cultivation of microorganisms from EMBRAPA bank (*Aspergillus sp.*), for the production of cellulases and xylanases, using non-conventional triphasic reactors.
- Physical-chemical pre-treatment of bagasse and characterization of the resulting biomass: Production of substrates for bioethanol production fermentation of hexoses and of pentoses.
- Determination of (sub-)optimal bioreactor operational conditions for fermentation of hexoses using free and immobilized enzymes.
- Assessment of the production of ethanol from hemicellulose using free and immobilized enzymes.

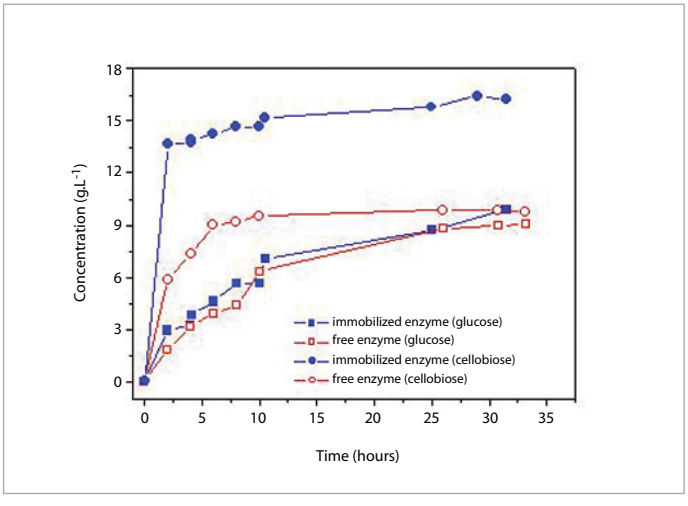

Figure 2. Assessing the performance of immobilization of cellulases. Immobilized enzyme load: 15 $U_{FP}.g^{-1}_{cellulignin}$. Free enzyme load: 5 $U_{FP}.g^{-1}_{cellulignin}$. All assays: 47ºC 0-10h and 37ºC until 10-33h, pH 5.0

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AN INTEGRATED PROCESS FOR TOTAL BIOETHANOL PRODUCTION AND ZERO CO_2 EMISSION

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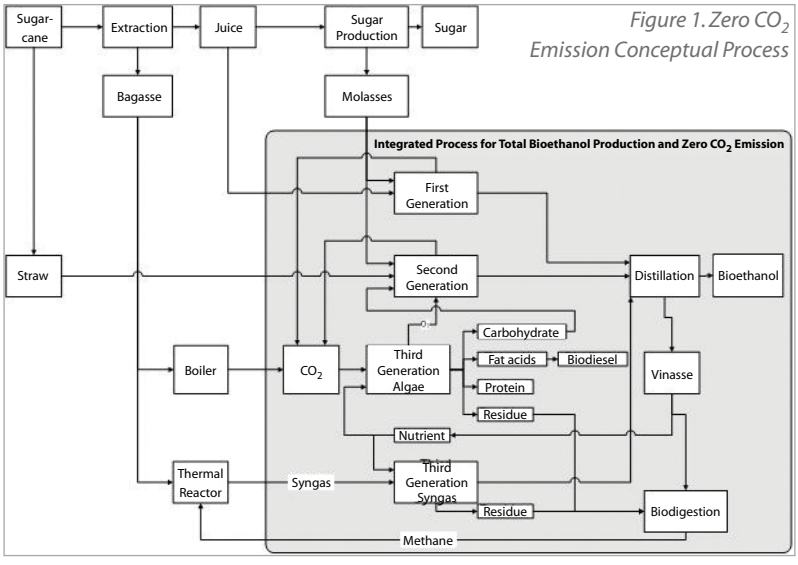

Figure 1. Zero CO_2 Emission Conceptual Process

Increasing oil prices and global concern about climate change motivate the investigation of more efficient means of bioethanol production. An integrated process is proposed in this Thematic Project, aiming to maximize the productivity of bioethanol from sugarcane molasses, bagasse and straw, which give rise to, respectively, first and second generation bioethanol. The Greenhouse Gas carbon dioxide, produced in this two ethanol generations processes, is proposed to be used in the production of a third generation of ethanol, which comes both from algal/bagass biomass and catalytic transformation or biological fermentation of synthesis gas. This challenging integrated process has the major appeal of not emitting carbon dioxide and makes the best of the carbon-containing material for producing ethanol, turning it, when technically and economically feasible, a milestone for improving Brazilian bioethanol competitiveness.

The specific objectives of this project look for ways of turning this integrated process into a technically and economically feasible one, investigating new technologies for each part of processes, covering first, second and third generation aiming anhydrous low bioethanol production. In this way, it will be studied alternative fermentation processes (extractive units), eco-efficient pretreatments as a part of an integrated bioethanol from sugarcane bagasse and straw, development of high-performing enzyme formulations and process techniques of both enzymatic hydrolysis and fermentation, coupled with the development of suitable yeast to ferment sugarcane biomass carbohydrates. In addition, the possibility of microalgae consuming CO_2 from alcoholic fermentation, constituting energy generation cycle with environment protection and production of bioethanol through the synthesis gas, which is obtained from biomass will be investigated. Besides that, multiple effect operation of the distillation columns of the process will be studied in order to reduce steam consumption on reboilers and compared to alternative strategies, including the hybrid ones. The study of alternative entrainers (ionic liquids and hyperbranched polymers) for the extractive distillation process for anhydrous bioethanol production will be carried out. Process modeling and simulation, either of single units or for the large scale plant will be used as a tool for process evaluation and decision taking. Process optimization will be considered to extract the best yield of each routes so that quantitative discrimination will be possible. In any case, methodologies that may be necessary whenever a re-estimation of parameters is required and among other tools software sensors based on Artificial Neural Network will be developed to infer concentrations of biomass, bioethanol and substrate from secondary measurements, such as pH, turbidity and CO_2 flow rate. The global processes evaluation will make use of the experimental data collected in the experiments, industrial data and information and together with process simulation trough commercial simulators and tailor made softwares. All the routes will be evaluated in the optimal conditions achieved by a set of suitable optimization algorithms including the deterministic and stochastic based ones.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The project proposed concept

This research project aims a totally integrated bioethanol production process, in order to improve the productivity of existing ethanol generation (sugarcane molasse fermentation), the so-called First Generation Bioethanol, to develop suitable processes for improving the Second Generation Bioethanol (from biomasses) and to investigate the viability of the Third Generation Bioethanol, which is produced from algal/bagasse biomass or from catalytical or biological fermentation of synthesis gas. The Third Generation Bioethanol has a major appeal of consuming carbon dioxide produced in the First and Second Generation processes, causing the great impact of almost zero CO_2 emission within the whole integrated process. The improvement of the energy intensive processes that constitute the distillation units are also objective of study in the present project and the proposal of alternatives procedures will be evaluated, including the hybrid configurations. *Figure 1* depicts a schematic diagram of the integrated process for bioethanol production.

Justification expected results

The interest in biotechnology-based production of fuels tends to increase with the concern about exhaustion of fossil fuels and the increase in their price. The world meetings make clear that policies for renewable energy are essential to achieve sustainable development in a broad sense. Environmental protection, job creation, alleviation of external debts in developing countries and security of supply are some of the key issues to mention. In Brazil, the sugarcane industry keeps the greatest commercial energy production in the world with bioethanol and the almost complete use of sugarcane bagasse as fuel. In addition to growing sugarcane and processing it to produce bioethanol and electricity, new biorefineries in Brazil should focus on marketing conventional bioethanol, its associated agricultural assets and co-generation plants, as well as making use of the acquired data and experience to contribute to research aimed at developing next generation biofuels. Thus, it is essential to implement a research program for the integrated production of bioethanol, where studies will be focused on improving the first generation bioethanol, the bioethanol from lignocellulosic feedstocks (second generation bioethanol) and mixed alcohols produced from synthesis gas, Substitute Natural Gas (SNG), and H_2 (third generation bioethanol). Although first-generation biofuels have the potential to replace fossil fuels as the main source of energy supply, its production is surrounded by issues like effects on global food supply and tropical forests destruction. Instead, second and third generation bioethanol offers the advantage of disconnecting the biomass from the food supply. These approaches have a better lifecycle and carbon footprint than sugarcane bioethanol. However, there are challenges and obstacles such as cost, technology and environmental issues that need to be overcome. Hence, the introduction of new processing integrated technologies is crucial in promoting and implementing bioethanol effectively and subsequently turning it in an environmentally, as well as economically, feasible source of energy.

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DEVELOPMENT OF β-GLYCOSIDASES DESIGNED TO IMPROVE THE EFFICIENCY OF NONCOMPLEXED CELLULASE SYSTEMS

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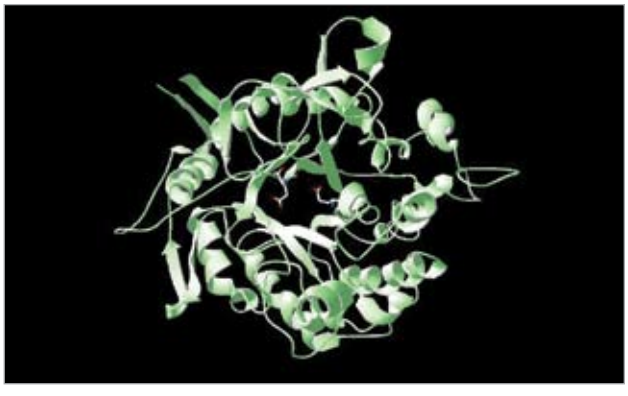

Figure 1. Homology model of Sfβgly. Active site residues are represented as sticks

The rate of the enzymatic hydrolysis of cellulose decreases along the reaction time, which represents a drawback for the productivity of this process. One of the factors causing that problem is the cellobiose inhibitory action upon the "cellulases" (endoglucanases and cellobiohydrolases) that catalyze the hydrolysis. This project aims to stabilize the rate of the enzymatic hydrolysis of cellulose by developing β-glycosidases designed to reduce the cellobiose inhibitory effect on the "cellulases". Such development will be based on the β-glycosidase (cellobiase) from the fall armyworm *Spodoptera frugiperda* (Sfβgly) and the "carbohydrate binding domain" of the endoglucanase EngXCA from *Xhantomonas axonopodis pv citri* (CBMXAC).

Firstly, a chimeric protein resulting from the fusion of Sfβgly and CBMXAC will be assembled. The targeting of Sfβgly to the surface of the cellulose fibers (due the presence of a CBM) could decrease the cellobiose concentration directly in the microenvironment of action of the endoglucanases and cellobiohydrolases. Thus the action of Sfβgly-CBM could reduce the cellobiose inhibitory effect and sustain a high activity of endoglucanases and cellobiohydrolases for a longer time.

This project also intends to improve the participation of the β-glycosidases in the cellulose hydrolysis by selecting mutant Sfβgly that presents high hydrolytic activity upon cellobiose. Libraries of random mutant Sfβgly will be generated and screened based on the ratio of activity upon cellobiose *versus* synthetic substrates. Finally, amino acid residues networks involved in the determination of Sfβgly substrate specificity and catalytic activity will be identified by using structural analysis, site-directed mutagenesis studies and enzyme kinetic experiments.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Production of the chimeric protein Sfβgly-CBM

The DNA segment coding for the CBM (0.4 kb) of the endoglucanase EngXCA from *X. axonopodis* (AE011689) was amplified taking genomic DNA of that bacteria as template, whereas the segment coding for Sfβgly (AF052729; 1.4 kb) was amplified from a cDNA libray of the *S. frugiperda* midgut. Following that, segments coding for CBMXAC and Sfβgly were fused by "overlapping pcr", which generated a product (1.8 kb) coding for the chimeric protein Sfβgly-CBM. This product was cloned into the vector pAE and this construction was introduced in BL21DE3 bacteria. In the next steps Sfβgly-CBM will be produced as recombinant protein and purified by Ni-binding chromatography. The catalytic activity of Sfβgly-CBM will be tested using p-nitrophenyl β-glycosides, whereas its cellulose-binding activity will be verified using avicel.

Enhancement of the cellobiase activity of Sfβgly

The search for Sfβgly mutants exhibiting high activity upon cellobiose was initiated by constructing libraries by random mutagenesis. A vector pCAL- Sfβgly was used as template according instructions of the kit GeneMorph II EZ Clone. In the next steps the library will be screened using a high-throughput procedure based on the recombinant expression and enzymatic assays in 96-well plates.

Identification of interaction networks in Sfβgly

In order to identify amino acid residues networks involved in the determination of substrate specificity and catalytic activity the tertiary structure of Sfβgly was represented as a graph and its central hubs were identified. In the next steps 10 of these central hubs will be separately removed by site-directed mutagenesis. The effect of these deletions on the Sfβgly substrate specificity and catalytic activity by enzyme kinetic experiments.

Figure 2. Amplification of the segments coding for CBMXAC (0.4 kb), Sfβgly (1.4 kb) and Sfβgly-CBM (1.8 kb), respectivelly

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BIOFUEL PRODUCTION BY PHOTOCHEMICAL CRACKING OF VEGETABLE OILS EMPLOYING AROMATIC IMIDES SUPPORTED ON MESOPOROUS SILICATES AS SENSITIZERS

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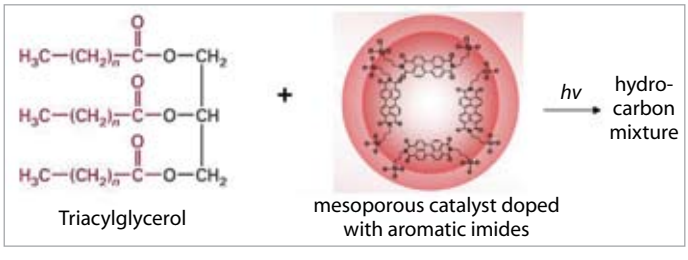
Catalytic cracking is a key reaction in the petrochemical industry, allowing the conversion of high molecular weight hydrocarbons into low molecular weight fuel. Up to the years 1930, the cracking reaction was performed by thermal methods, but since then, with the introduction of the zeolites, which are microporous heterogeneous catalysts, catalytic cracking has become the most widely used method. More recently, it has been recognized that catalytic cracking of vegetable oils could be used to obtain biofuel, as an alternative to the transesterification reaction. The use of photochemistry in cracking reactions, however, has remained largely unexplored. In the present proposal, the development of photochemical catalysis as a method for the cracking of vegetable oils will be pursued, using principles of nanotechnology to design nanostructured photocatalytic systems. For this goal, aromatic imides, such phthalimides, 1,8-naphthalimides, 1,4,5,8-naphthalenediimides and 3,4,9,10-perylenediimides, will be employed as photosensitizers. Our group has great expertise in the photochemistry of these compounds. When excited by light, these compounds generate a variety of free radicals, which are expected to stimulate the cracking reaction, which is a radical chain reaction. The imides will be immobilized by covalent grafting onto the surface of silicates MCM-41 and SBA-15, which are mesoporous nanostructured materials synthesized in the presence of surfactant micelles. The modified particles will be irradiated with a UV lamp and with natural sun light, in the presence of different vegetable oils. Mesoporous materials with different loads of the organic dye will be tested, containing either a single imide or a mixture of different imides.

Figure 1. (A) Photographs showing the mesoporous catalyst MCM-41 before (left) and after (right) modification with the photosensitizer PDI. (B) Optical micrograph of the modified mesoporous catalyst, registered with an inverted fluorescence microscope, showing the strong red emission of the incorporated dye

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

In the first year of the project, the mesoporous catalysts doped with the photosensitizers were prepared by covalent grafting of 3,4,9,10-perylenediimides (PDI) onto the walls of molecular sieves MCM-41 and SBA-15. The mesoporous materials were first treated with 3-aminopropyltriethoxysilane (APTES) in anhydrous toluene, generating amine-containing surfaces. The amine-containing materials were then reacted with 3,4,9,10-perylenetetracarboxylic dianhydride (PTCA), generating surface-grafted PDI. The samples showed the red color which are typical of the PDI dyes (*Figure 1A*). The new materials, designated as MCMPDI and SBAPDI, presented absorption and emission spectra corresponding to weakly coupled PDI chromophores, in contrast to the strongly coupled rings usually found in solid PDI samples. The materials showed a red fluorescence, which could be observed by the naked eye under UV irradiation or with a fluorescence microscope (*Figure 1B*). The next step is the test of the catalytic activity of the new materials for the cracking of vegetable oils, using a thermogravimetric analyzer coupled with mass spectrometer. This system allows *in situ* analysis of the hydrocarbon mixture obtained after thermal degradation of the oil. The system will be tested in the dark and under UV irradiation.

Graphical abstract. Catalytic cracking of vegetable oils by a mesoporous catalyst doped with an aromatic imide



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RESEARCH AND DEVELOPMENT AIMING AT THE INTEGRATED EXPLOITATION OF SUGARCANE BAGASSE FOR THE BIOTECHNOLOGICAL PRODUCTION OF LIGNOCELLULOSIC ETHANOL

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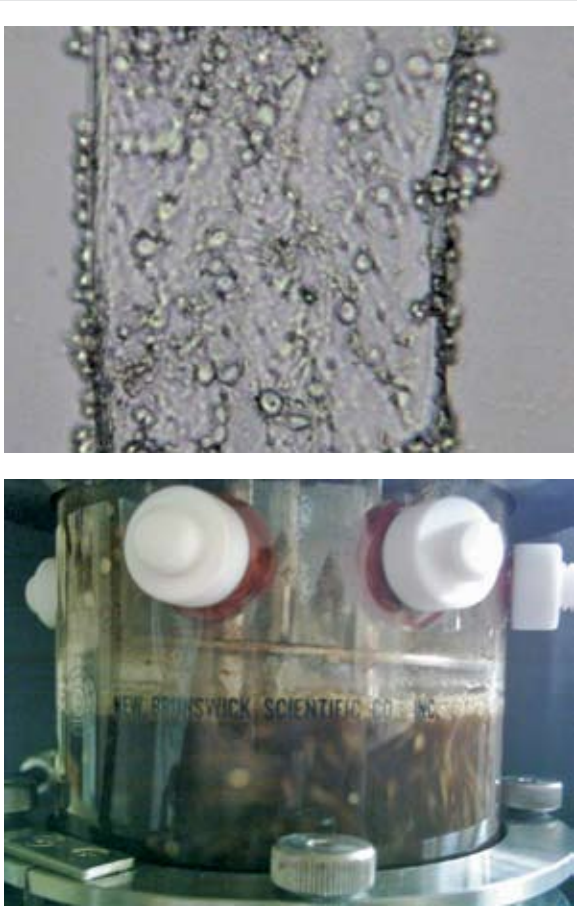


Figure 1. Growth of xylose-fermenting yeasts in bagasse fiber and fermentation of sugarcane bagasse hydrolysate in a batch bioreactor aiming bioethanol production

This project is a collaborative research within the BIOEN-FAPESP/FAPEMIG call between the Biotechnology Department of the Lorena School of Engineering (EEL/USP), Physics Department of the Federal University of Juiz de Fora (UFJF), Microbiology Department of the Federal University of Minas Gerais (UFMG) and Microbiology Laboratory, São Paulo State University (UNESP Rio Claro).

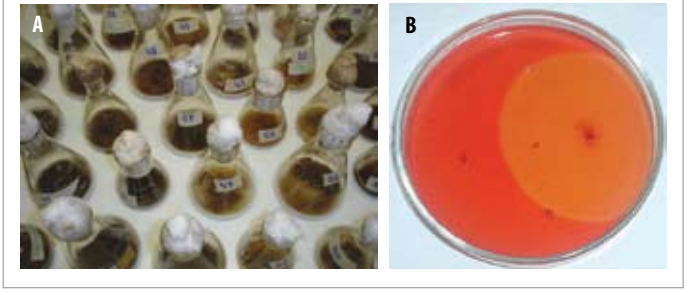
The project aims at the fractioning of sugarcane bagasse in its main components (cellulose, hemicellulose and lignin) for their use in the production of ethanol. The acid hydrolysis will be used to remove the hemicellulosic fraction followed by alkaline hydrolysis. The enzymatic hydrolysis of the cellulose fraction will be performed. The hydrolysates will be characterized by advanced spectroscopic techniques as Raman scattering, infra red absorption with Fourier transformation (FTIR), absorption in the near infra-red ray (NIR), thermal lens and photo-acoustic. The use of these techniques is interesting due to their nondestructive character, possibility of *in situ* measurements and further development of compact prototypes. In the further stage, the xylose and glucose rich hydrolysates will be properly treated by detoxification methods and used as fermentation media for the production of ethanol by xylose-fermenting yeasts and *S. cerevisiae*, respectively. Xylose-arabinose fermenting species isolated from the Atlantic Rain Forest, Amazon Forest and Brazilian Cerrado ecosystems will be tested. Yeasts inhabiting rotting-wood substrates will be collected and tested for xylose-arabinose fermentation. This project aims at finding new species from these Brazilian ecosystems capable to be used in industrial processes.

In all the involved unitary operations in this study, conditions will be optimized by experimental design and data analysis by means of appropriate statistical methodologies. Obtained results will allow establishing advanced technologies and innovations in order to extend the national and international competitiveness of "bioenergy" to reach second generation technologies for alcohol program. It will allow the formation of teams and cooperation among the participant institutions for training and exchange of knowledge.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

- Generate scientific knowledge with approaches related to the integrated use of sugarcane bagasse for bioethanol obtainment;
- Fractionation of sugarcane bagasse in a lab scale reactor under controlled acid hydrolysis;
- Screening of new microorganisms able for utilization of xylose-arabinose as carbon sources in bioprocesses aiming ethanol production and identification of new selected yeasts by physiological and molecular methods (PCR);
- Isolation of yeasts with cellulolitic activity and preservation;
- Comparison of different strategies for sugarcane bagasse hydrolysate detoxification;
- Fermentation of sugarcane bagasse hemicellulosic hydrolysate by the new xylose-fermenting selected strains.
- Development of new analytical methods based on spectroscopic principles for characterization of sugarcane bagasse, hydrolysates and ethanol produced by fermentation process. It is expected the characterization measurements of these materials by Fourier Transform Infrared Spectrometry (FTIR), Near infrared spectroscopy (NIR), Raman spectroscopy, thermal lens and photo-acoustic.
- Development of a spectroscopic data processing software using the Principal Component Regression (PCR) and Partial Least Square (PLS) methods to be inserted in programmable microcontrollers (PIC-Programmable).

Figure 2. Screening of new xylose–arabinose fermenting yeasts from Amazon, Atlantic Rain Forest and Brazilian Cerrado ecosystems and cellulolytic activity of selected isolated strains



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AISE – THE SWEET AND BITTER SIDES OF THE SUGARCANE. AN INTEGRATED SUSTAINABILITY ASSESSMENT FOR THE BRAZILIAN ETHANOL CONTEXT

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The growing international discussion on the role of global warming and renewable energy boosts up once again the interest for bio-fuels. But there is, yet, a significant anxiety of the society about the present patterns of sugarcane cultivation and ethanol production impact balance. The current modus operandi of public policy formulation and implementation, based on punctual and setorized socioenvironmental impact assessment, limits and hides complex productive system functioning essential factors, as observed for the sugarcane ethanol context, enlarging ethanol production sustainability compromising risks. One of the knowlegde gaps is exactly in the methodology design, that will make it possible to integrate the various sustainability elements, following principles of sustainability tailored to the sugarcane ethanol.

Photos by Joviniano Pereira da Silva Netto

The challenge is exactly in the concept validation process with the stakeholders and the methodology proposal in a way that it will be incorporated by institutions and society in their management and decision making process. It is part of this challenge the study of the existing methodologies and the possibilities of interlinkage and integration among them, what means, turn them more powerful and pragmatic to face the inherent complexity of the sustainability of the sugarcane ethanol context.

Therefore, this research has the general aim of developing and applying an ISA (Integrated Sustainability Assessment) Methodology for sugarcane ethanol to the state of São Paulo, Brazil.

The AISe group includes researchers from several institutions:

- University of São Paulo (USP): São Carlos Engineering School (EESC/USP), School of Arts, Sciences and Humanities (EACH/USP), Luiz de Queiroz Agriculture School (ESALQ/USP) and Ribeirão Preto School of Philosophy, Sciences and Literature (FFCLR P/ USP);
- Institute of Agriculture Economics (IEA);
- Institute for Agricultural and Forest Management and Certification (IMAFLORA);
- Brazilian Agricultural Research Corporation (EMBRAPA);
- Michigan University.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The main expected results of this research are: (i) scientific discussion about the concept of sustainability applied to sugarcane ethanol context, with the engagement of stakeholders; (ii) proposition of a sugarcane ethanol ISA Methodology conception, available for use by the governmental and nongovernmental institutions actuating in the bioenergy area and also for institutions involved with the BIOEN Program; (iii) the basis for the construction of a software on the continuation of this project in the following years; (iv) governmental and nongovernmental institution manager capacity building relatively to ISA methodology of sugarcane ethanol; (v) research team consolidation.

The organized events are:

- I Mesa-redonda sobre etanol de cana-de-açúcar: migração e meio ambiente – April/2008.
- II Mesa-redonda sobre etanol de cana-de-açúcar: um diálogo acerca da sustentabilidade – April/2009
- I Workshop AISE – Avaliação Integrada da Sustentabilidade do Etanol – Dec/2010
- Bioen Workshop on Integrated Sustainability Assessment for Ethanol Context – April/2010

The concluded dissertations and monographs are:

Monographs:

- Discussão sobre as considerações metodológicas relativas à análise de inventário de avaliação de impacto do ciclo de vida para o uso de agrotóxicos
- A atuação do setor governamental na gestão ambiental local no contexto do etanol brasileiro

Dissertations:

- Uso da biomassa da cana-de-açúcar para geração de energia elétrica: análise energética, exergética e ambiental de sistemas de cogeração em sucro-alcooleiras do interior paulista
- Sistema Municipal de Meio Ambiente e produção de etanol de cana-de-açúcar no Estado de São Paulo: estudo de casos em Brotas e Araraquara
- Instrumentos de intervenção governamental e postura ambiental empresarial: uma análise da agroindústria canavieira do Estado de São Paulo

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