BIOEN

BRAZILIAN RESEARCH ON BIOENERGY

- BIOPHYSICS
- BIOCHEMISTRY
- GENETICS
- MICROBIOLOGY
- INTERDISCIPLINARY

FAPESP

BIOENERGY: THE BRAZILIAN EXPERIENCE

Bioenergy is an important clean energy alternative. The Brazilian experience has shown that sugarcane is a highly sustainable crop for bioenergy production

Brazil is outstanding as the world's most intensive user of bioethanol as an alternative to gasoline for powering transport. Total bioethanol production in 2014/15 corresponds to 28.4 billion liters in 366 plants. In 2015/16 around 58 per cent of the 590 million tons of sugarcane will be used for ethanol and 42 per cent for sugar production. The total sugarcane planted area in Brazil is around 10 million hectares (ha). This accounts for only around 6-7 per cent of total area devoted to agriculture and 1.1% of the country's total area.

CLEAN AND CHEAP ENERGY

Brazil and the USA produce more than 80 per cent of world's ethanol. Brazilian bioethanol production costs are the cheapest in the world. In addition to low production costs, ethanol produced from sugarcane in Brazil has another important advantage: in Central-South Brazil, only 1 unit of fossil energy is consumed for each 9-10 units of energy produced by ethanol from sugarcane. The use of ethanol as a biofuel also reduces carbon emissions: when ethanol is used in substitution to gasoline, there is a 76% reduction in GHG emissions with a simultaneous decrease in SO_2 emission. Since 2003, Brazil's use of sugarcane ethanol has avoided the emission of 242 million tons of carbon dioxide.

THE BRAZILIAN MODEL

Sugarcane was introduced in Brazil in 1532 by the Portuguese and it has been cultivated here ever since. The research that started in the first half of the 20th century has gradually improved this crop, with the development of new varieties of sugarcane that enhanced the yields of both culm and sucrose. During the 1970s, the "Brazilian model" of producing sugar and ethanol together brought important technical improvements and enabled 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 2014, 88.2% per cent of the 2.9 million cars and light vehicles sold in Brazil 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 also of the overall energy content (biomass yield).

Traditionally, 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 biomass (bagasse and leaves).

BIOEN FACTS AND FIGURES

US$ 167 million	**research expenditures by FAPESP and partners**
13	**co-funding partners**
7	**private companies co-funding projects**
467	**scholarships –** 89 ongoing and 378 completed
206	**research projects –** 66 ongoing and 140 completed
300+	**researchers involved**
21	**fields of knowledge**
920+	**scientific publications**
17	**patents filed**

THE ENERGY-CANE

With the possibility of cellulosic ethanol production, scientists 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 or other liquid fuel, 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 7,000 to about 12,000 liters per hectare-year, i.e., between 60 and 70 per cent.

In 2014, Brazil burned bagasse to produce energy equivalent to 20,823 GWh. 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 at increasing even further the energy supply from bioelectricity. Also, most of the straw still remains in the field after sugarcane harvest and can be, at least partially, transformed in energy. Bioelectricity from sugarcane can contribute to 18% of the country's energy demand. Additionally, sugarcane offers options in development: alternative routes to produce liquid fuels from lignocellulosic materials for road, maritime and aviation transportation could open new possibilities for an industry that is already energy-efficient.

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.

FAPESP'S BIOENERGY PROGRAM – BIOEN

To respond to the increasing need for R&D in the area of bioenergy the São Paulo Research Foundation (FAPESP) created a Bioenergy Program (BIOEN). FAPESP is one of 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 is organized in five divisions: Biomass, Biofuel Technologies, Biorefineries, Engines, and Impacts and Sustainability.

Over 300 researchers support BIOEN activities with funds in the order of US$ 167 million. BIOEN is increasingly multidisciplinary including projects from 21 areas of knowledge. This highlights the broad scope of questions addressed. The BIOEN Program consolidated the community in an active network of experts leading 206 research projects and 467 scholarships in 21 institutions in the State of São Paulo, in collaboration with other institutions in Brazil and in 29 countries. Since 2008, BIOEN has generated more than 920 scientific publications. Additionally, more than 300 thesis and dissertations with important contributions for the advancement of science and the industry have been concluded. This represents an important step in generating human resources to increase the potential and the number of qualified professionals working in this field.

BIOEN

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

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FAPESP Process 2008/56256-5 | Term: Jun 2009 to May 2013 | Thematic Project
co-PI: Andre Luis Ferraz

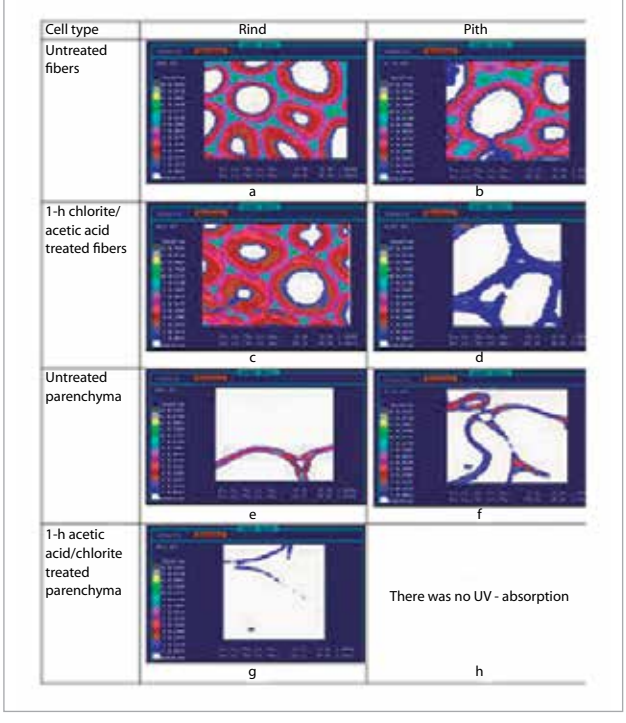

Figure 1. Scanning UV-micrographs of 1µm transverse sections of sugar cane cells. Appropriate software translates the absorptions intensities at 278 nm (shown in the left of the image) into multiple colors to illustrate the lignin distribution in the biomass tissues. The image clearly indicates the cell corners as the region with the highest absorption (colored with light- and dark green), followed by the middle lamella (colored with light green to pink) and by the secondary cell wall (colored with pink to dark blue) (Reproduced from Siqueira et al. 2011)

Lignocelulosic biomass is recalcitrant to enzymatic digestion because terrestrial plants develop an efficient manner to grow upward and resist the microbial degradation of the polysaccharides contained in their cell walls. The complex cell ultrastructure, varied tissues, and the composite characteristic of the cell walls are among the several factors explaining the recalcitrance of lignified plants. Mapping the macromolecular components in the cell walls has proved to be useful to understand the varied recalcitrance of different biomass tissues. Available data indicate that lignin and hemicellulose greatly affect the final digestibility of the lignocellulosic materials. Removal of these components from the cell walls with varied pretreatments or even using lignin- and/or hemicellulose-depleted plants indicate that a critical characteristic of the cell wall to be digestible is to present most as possible available cellulose. Saccharification of sugarcane bagasse based on the enzymatic hydrolysis of forthcoming plants down regulated on lignin biosynthesis or prepared by using a selective chemical step followed by mechanical fiberizing was developed at mild conditions. These bagasse samples served 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. Cellular ultraviolet microspectrophotometric evaluation of the samples suggested that in the parenchyma and vessel cell walls, the hydroxycinnamic acids were linked to the lignocellulose backbone mainly through alkali-labile ester linkages. By contrast, the C-4 positions of the aromatic rings of the hydroxycinnamic acids contained in fiber cell walls should be etherified to lignin. The pretreatment caused intense delignification in the majority of the internode regions. The outermost regions were the most resistant to lignin and hemicellulose removal (*Figure 1*). Enzymatic hydrolysis of the pretreated samples indicated that the outermost fraction and the rind were recalcitrant regions, whereas the pith-rind interface was less recalcitrant.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Sugarcane bagasse with different lignin contents were prepared by chlorite delignification providing more accessible cellulose, resulting in higher hydrolysis rates. More than 90 % of the cellulose was converted into glucose by removing 63 % of the initial lignin, while all cellulose was hydrolyzed after removing 72 % of the lignin. Combining the effect of lignin removal and the addition of β-glucosidases to the reaction medium, less lignin needed to be removed to achieve similar hydrolysis levels. The UV microspectometry of lignin moieties in different cell types of mature sugarcane samples showed the highest UV absorbance in the cell walls of vessels followed by fibers and then parenchyma (*Figure 2*). Highly lignified fibers from the rind region of the internode become less recalcitrant as a function of the lignin and hydroxycinnamic acids removal. Untreated parenchyma cells from the pith region were promptly hydrolyzed by commercial cellulases indicating that the action of the cellulolytic enzymes was not restrained by the aromatics occurring in the pith parenchyma, but it was strongly controlled by the high lignin content present in the fiber cell walls from the rind region of the internode. The chlorite treatment led to significant removal of hydroxycinnamic acids and lignin of the rind cells in vascular bundles, resulting in a significant enhancement of the cellulose conversion by commercial cellulases. The total hemicellulose content in sugarcane bagasse increased from pith toward rind, therefore the sum of hemicellulose and lignin was a key factor to explain the varied recalcitrance of the different tissues. The alkaline/sulfite pretreated material was less recalcitrant to the enzymatic hydrolysis, since 85 % of cellulose conversion was obtained. The sulfonic acid groups turn the residual lignin less hydrophobic allowing an increased capacity of water retention by the fibers. The swollen fibers become more porous, facilitating the enzyme permeation toward the secondary walls of the pretreated material. The cellulotytic enzymes apparently do not adsorb irreversibly to the lignin in this type of pretreated material, which diminishes the enzyme load required for efficient hydrolysis as well as turn the enzymes recycling more feasible. These combined effects have been claimed to bring low enzyme consumption and costs in this type of process.

MAIN PUBLICATIONS

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SECRETION OF HETEROLOGOUS GLYCOPROTEINS IN ASPERGILLUS: EFFECT OF GLYCOSYLATION PATTERN IN FUNCTIONAL PARAMETERS OF GLYCOSYL HYDROLASES

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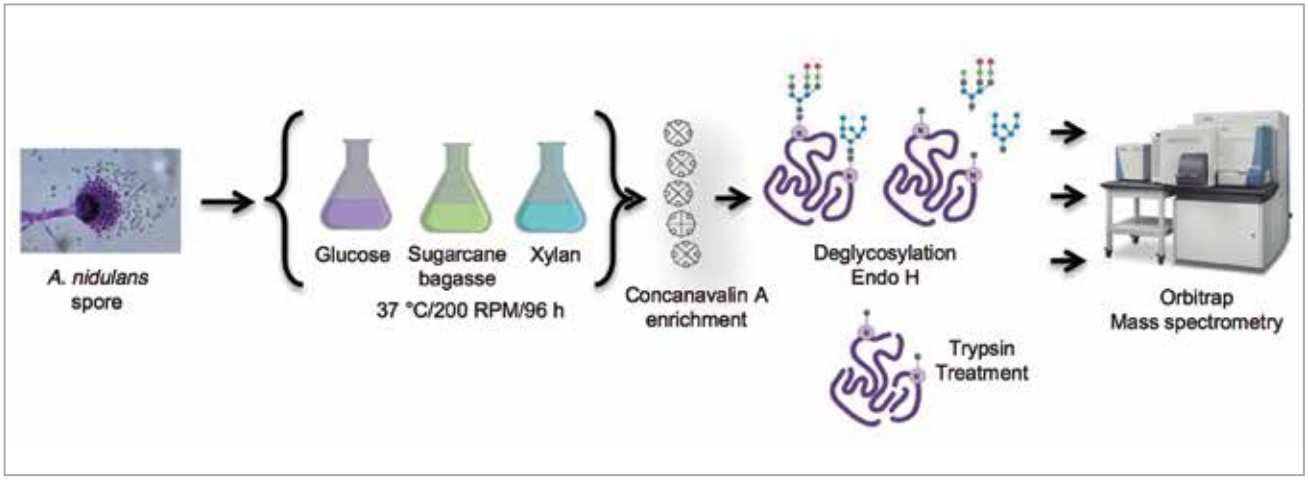

Figure 1. Glycoproteomics pipeline. This approach was applied to the analysis of total secretome of A. nidulans grown in glucose, xylan and sugarcane bagasse. The aim is to identify N-glycosylation sites in Carbohydrate-Active Enzymes (CAZymes) studying possible patterns. Comprehensive analysis of protein glycosylation processes in A. nidulans will assist in a better understanding of glycoprotein structures, profiles, activities and functions.

The use of renewable sources in obtaining fuel becomes an important alternative, by generating fewer pollutants and allows the sustainable development of economy and human society. Alternatively, the use of lignocellulosic biomass, mainly composed of cellulose, hemicellulose and lignin, is a consensus in worldwide, since it is the most abundant renewable energy source in the Earth.

Due to the complexity of plant biomass, which is rich in glycoconjugates, oligo- and polysaccharides, a wide variety of enzymes should act in conjunction to degrade this type of biomasses. The Carbohydrate-Active enZymes (CAZymes) participate in breakdown, biosynthesis or modification of the plant cell wall compounds. In general, CAZymes are structurally constituted by a catalytic domain and some families have an additional carbohydrate-binding module (CBMs). Based on structural and functional features, the CAZy database currently cover five enzyme classes such as glycoside hydrolases (GHs), glycosyltransferases (GTs), polysaccharide lyases (PLs), carbohydrate esterases (CEs), and auxiliary activities (AAs).

Heterologous protein expression in filamentous fungi shows interesting advantages compared to other hosts. First, filamentous fungi secrete proteins in large quantities what results in high production levels and facilitates their purification and characterization. Second, most genes from fungi have introns and these organisms are able to recognize and process them correctly. Finally, several fungal proteins are glycosylated, and the protein expression in another filamentous fungus results in a glycosylation pattern that is similar to that of the native fungal enzyme of interest.

We will apply proteomic and transcriptomic approaches to understand how the *A. nidulans* adapts to the high expression and secretion of heterologous proteins by global analysis. We compared four strains of *A. nidulans*, with wild type, an empty plasmid-transformed strain and two heterologous strains producing a GH51 arabinofuranosidase (AbfA) and GH7 celobiohydrolase (CbhI) cloned from *A. fumigatus*. Moreover, there are few studies mapping the global N-glycosylation of CAZymes in filamentous fungi. In this study we will map the profile of N-glycoproteins in *A. nidulans* secretomes trying to understand the patterns of N-glycosylation in CAZymes. The most common and frequent N-glycosylated motifs, an overview of CAZymes glycosylation and the number of mannoses found in N-glycans will be analyzed with this data. Comprehensive analysis of protein glycosylation processes in *A. nidulans* will assist in a better understanding of glycoprotein structures, profiles, activities and functions. This knowledge can allow the optimization of heterologous expression and protein secretion using *A. nidulans* as a model host.

MAIN PUBLICATIONS

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Segato F, Damasio ARL, de Lucas RC, Squina FM, Prade RA. 2014. Genomics review of holocellulose deconstruction by aspergilli. *Microbiol Mol Biol Rev.* **78**: 588-613 (cover paper).

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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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FAPESP Process 2008/52197-4 | Term: Aug 2009 to Jan 2014 | Thematic Project
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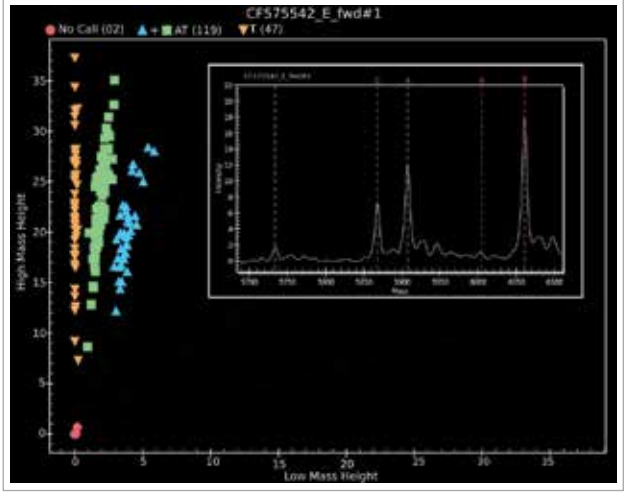

Figure 1. Example of results obtained with the SNP technology. Upper right: results for a single individual, showing the results for two loci; each peak corresponds to a nucleotide (A and C for the first, A and T for the second, that is homozygous). Left: genotyping of the segregating population, showing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

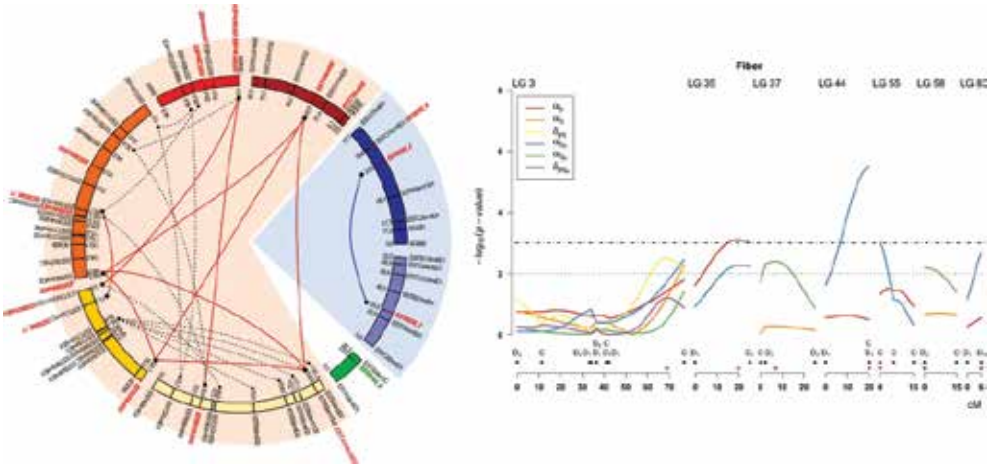

Figure 2. Example of mapping results. Left: Eight linkage groups obtained from markers with single (names in black), double (red) and triple dose (green). Lines are connecting the homology groups based on properties of the markers (EST-SSR and EST-RFLP) (dotted lines) or on loci with more than one copy 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. Glaucia 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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GENE EXPRESSION PROFILE AND CARBON ISOTOPE DISCRIMINATION IN SUGARCANE GENOTYPES UNDER WATER DEFICIT STRESS

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FAPESP Process 2008/57923-5 | Term: May 2009 to Apr 2012

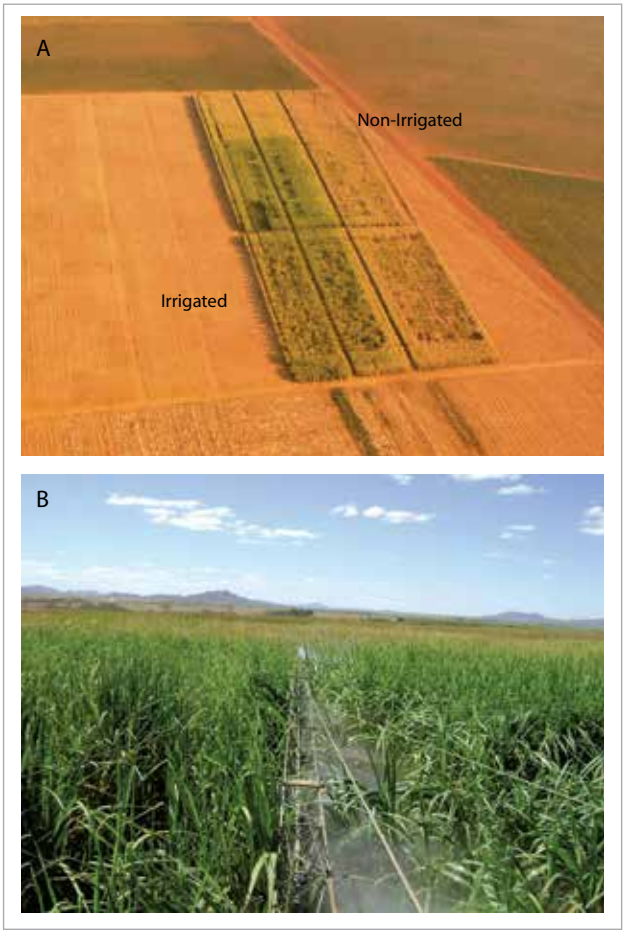

Figure 1. Field (irrigated and non-irrigated at "Cerrado"). A. Bird's-eye view. B. A general overview of the experiment

Sugarcane (*Saccharum spp.*) is major crop in Brazil as feedstock for the sugar and ethanol industries. To attend the increasing ethanol demand, the sugarcane industry must expand the cultivated area, incorporating land from 'cerrado' and pastures, characterized by a dry winter with a long water deficit period. For the last 10 years, more than 80 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 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, promoting 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 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

The Brazilian sugarcane industry is expanding rapidly, particularly to drought-prone regions. We characterized tolerance by phenotyping and attempted to identify genes with roles in drought tolerance. From 100 genotypes, 10 were selected and evaluated for drought tolerance in a field trial in Goianésia and under greenhouse conditions. Based on morphophysiological evaluations, we identified two contrasting genotypes for response to drought stress: 'IACSP94-2094' showed enhanced features of drought tolerance (lower transpiration; maintenance of leaf water potential; and superior photosynthesis activity), limited in 'IACSP97-7065'. Gas exchange and photochemical parameters, carbon isotope discrimination ($\Delta^{13}C$), leakage of CO_2 (φ), and the enzymatic pathway involved in the transport of the C_4 acids from mesophyll to bundle sheath cells were further investigated in these cultivars. 'IACSP94-2094' displayed higher carbon discrimination and leakage (φ) during water stress. Quantitative gene expression profile and enzymatic activity consistently suggested the occurrence of the phosphoenolpyruvate carboxykinase (PCK) alternative during water stress in 'IACSP94-2094'. Leaf samples collected from these contrasting genotypes under irrigation or drought conditions in a field trial at two moments (early and after severe drought) were used to perform microarray analysis. From a set of 14,522 genes, 91 were differentially expressed between irrigated or non-irrigated treatments during early drought, whereas 576 were differentially expressed during severe drought between water treatments, from which 438 were differentially expressed between genotypes. 'IACSP94-2094' showed more changes in expression than 'IACSP97-7065' in genes from pathways associated with drought tolerance, such as oxidation/reduction, hormone metabolism, response to stress, and response to abiotic stimulus by gene ontology analysis. Leaf samples from the same genotypes grown in the greenhouse under similar treatments were used for gene expression profiling by RNAseq. Using the sugarcane assembled sequences as reference (43,141 genes), we identified 2,300 as differentially expressed. There was an important correlation between differentially expressed genes identified by microarrays and observed in RNAseq, with similar expression levels. Differentially expressed genes in 'IACSP94-2094' under stress were associated with photosynthesis, particularly light reaction centers and C4 decarboxylation.

Figure 3. Greenhouse trial. Instituto Agronômico de Campinas (IAC)

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METABOLOMICS AND PROTEOMICS OF SUCROSE ACCUMULATION IN SUGARCANE

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1. Characterization of the metabolome and proteome of leaves, internodes (juvenile and mature), at different stages of development. The sugarcane cultivar analysed was SP80-3280.

1.1 Characterization of the proteome of juvenile and mature internodes during sucrose accumulation at different stages of sugarcane development.

1.2 Cell wall proteomics of cells and young leaves and internodes from sugarcane.

1.3 Development of the methodology for isolation of intact nucleus from young sugarcane leaves for its proteomic characterization.

1.4 Continuation of the above project aiming to identify differences in the nucleus proteome of sugarcane plants exposed to water stress. The experiments were conducted in greenhouse conditions and following the water stress treatment, plants were left to recover, in order to identify proteins linked to recovery process.

1.5 Proteomics and metabolomics of sugarcane under water stress using the cultivar SP 80-3280 under field conditions

1.6 The second project on proteomics and metabolomics of sugarcane analysed the responses of two varieties of sugarcane, tolerant and susceptible, to water stress.

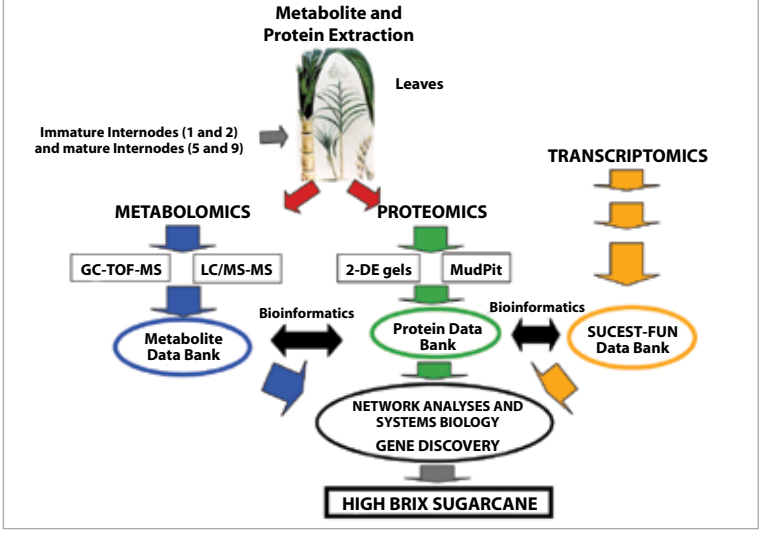

Figure 1. Experimental flow showing the main activities to be developed in the project

1.7 Field experiments (UFSCAR) in collaboration with the group of Prof. Glaucia Mendes Souza (*Sugarcane Signaling and Regulatory Networks* – Thematic project FAPESP 2008/52146-0). The aim of this project is to establish the metabolic profile of plants from the cultivar SP 80-3280, under field conditions.

1.8 Lipidomics of leaves of sugarcane using the technique of Maldi Imaging (IMS). This project is being developed in order to understand the lipid composition of sugarcane leaves.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The thematic project was important to establish several lines of research in our laboratory, such as proteomics and metabolomics of sugarcane. We were able to establish the techniques of quantitative proteomics and metabolomics. Besides, we had the possibility of training several undergraduate and graduate students (MS and PhD) as well as post-docs. We established a good scientific collaboration with Prof. Elizabeth Jamet from the University Paul Sabatier, of Toulouse-France, with whom we published the first article on cell wall proteomics of cell culture of sugarcane. Presently, we have submitted the second manuscript of cell wall proteomics of sugarcane leaves to BMC Plant Biology.

Certainly, the project is being continue with research lines started and in the following years we will be publishing more articles related to these topics.

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Submitted:

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DEVELOPMENT OF STRATEGIES FOR TRANSFORMATION OF GLYCEROL VIA BIOTECHNOLOGY AND CHEMICAL ROUTES

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FAPESP Process 2008/03620-1 | Term: Apr 2010 to Mar 2013 | PITE – Business partner: Braskem S/A

1,3-Propanediol (1,3 PDO) is a chemical compound of remarkable value in the synthesis of terephthalate polymers, cosmetics, lubricants, among others; 3-hydroxypropionaldehyde (3-HPA) is used for food preservation, polymer production and as a precursor to many chemicals, such as acrolein, citric acid and also of 1,3 PDO itself. Both compounds can be produced biologically by microrganisms via fermentation of glycerol, which is generated in excessive amounts as a byproduct of biodiesel production. The project goal is to obtain conversion of glycerol to 3-HPA and 1,3 PDO through two microbiological strategies. One is to perform genetic modifications in other organisms using genes from *Klebsiella pneumoniae* bacteria, which is one of the largest producers of 1,3 PDO. The other strategy is to improve the conversion of these products (mainly 1,3 PDO) by *Lactobacillus reuteri*, which is also a natural producer microorganism. Another objective set out in the project is the chemical modification of glycerol to generate ionic liquids, so-called green solvents. These compounds have relevance for the development of new materials replacing organic solvents, which are used in such synthesis reactions.

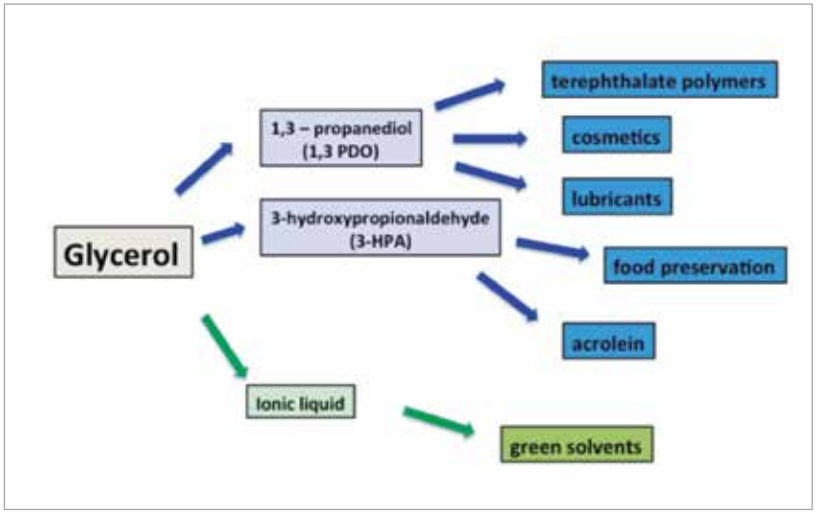

Figure 1. Glycerol from diesel waste may be converted by the microbiological (blue) or chemical (green) pathways to other sustainable products, as indicated

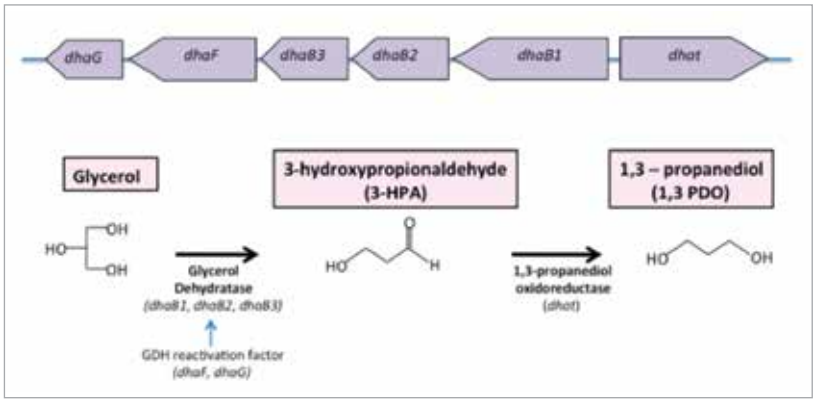

Figure 2. Glycerol assimilation through the fermentative pathway, showing the reductive pathway, producing the 3-HPA and 1,3 PDO. In this pathway, glycerol is reduced to 1,3-PDO by the successive action of glycerol dehydratase (GDH) (encoded by three dhaB genes) and 1,3 propanediol oxidoreductase (encoded by dhaT gene). On top, the genomic arrangement of dha genes present in Klebsiella pneumoniae is depicted; arrows indicate the direction of transcription. The genes refer to those found in K. pneumoniae (modified from Martins-Pinheiro et al, submitted to publication)

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The heterologous expression of genes from *K. pneumoniae* did not lead to the expected high production of 3-HPA or 1,3-PDO in *Escherichia coli*. Therefore, we focused our efforts on two alternative strategies to overcome the difficulties and develop a specific process, with several elements of originality, for the production of 1,3-PDO from glycerol. One of the strategies was to identify functional homologs from the synthetic route in prokaryote genomes through *in silico* analyses. These analyses revealed the evolution of the genes related to the glycerol metabolic route, as this was found to be present in several bacterial species and even in one Archaea species. They also converge with the project goals, identifying new potential genes that can be used for obtaining 1,3-PDO from glycerol. The results were submitted for publication. In the second strategy, the use of the bacteria *Lactobacillus reuteri* was employed with success by increasing the glycerol conversion factor and productivity of 1,3-PDO. We investigated the behavior of Lactobacillus reuteri ATCC23272 when cultivated in batch, repeated batch and continuous modes (chemostat process) during the conversion of glycerol into 1,3-PDO. The production of this product in repeated batch, with cell decantation, was reported for the first time; the results in the chemostat indicated a high productivity of 1,3-PDO (4.92 $gL^{-1}h^{-1}$), with a yield based on glycerol near the theoretical maximum. Moreover, 1,3-PDO productivity was observed to be directly related to the rate of glucose consumption and, because the chemostat process favors glucose uptake rate, it favored production. Thus, a novel process for 1,3-PDO production from glycerol was developed, yielding a patent claim.

Concerning the chemical route to produce ionic liquids, the first trials have shown the feasibility of synthesis using glycerol and 1-methylimidazole as starting materials. The step of preparing the precursor glycerol trimesilate was well established, but nucleophilic substitution with 1-methylimidazole resulted in a mixture of products derived from tri- and di-substitution in a ratio of 3: 2. Optimization of the reaction conditions produced the tri-substituted ionic liquid in higher purity and larger quantities (grams). The presence of a recurrent impurity indicated a possible thermal instability of the tri-substituted ionic liquid, the pyrolysis of which under controlled conditions indeed resulted in the production of another novel ionic liquid, formed by the elimination of the central methylimidazolium group. This thermal instability, not anticipated in advance, greatly limits the use of the tri-substituted ionic liquid in practical applications. Nonetheless, the new disubstituted propenyl ionic liquid, formed as the predominant pyrolysis product, could have interesting and unique properties. The polymerization of compound 4 could potentially form a much more stable polymeric ionic liquid derivative of polypropylene, a possibility not explored further due to the conclusion of the project.

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Patent Claim:

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CIRCADIAN RHYTHMS IN C4 GRASSES

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Figure 1. Sugarcane field in UFSCar (Araras – SP) in different times of the day: at dawn, in the middle of the day, at the end of the day, and at night. The circadian clock allows the plant to anticipate rhythmic changes, such as in light, temperature , and humidity

Plants have internal timekeeping mechanisms that increase their productivity in a rhythmic world. These mechanisms, called the circadian clock, allow plants to anticipate rhythmic changes in the environment, such as light and dark or warm and cold; to detect seasonal changes; and to organize their metabolism during a single day. One of the major challenges in the area is to fully understand how the circadian clock enhances the plant productivity. Arabidopsis thaliana with changes in their circadian clock accumulate less biomass, have less chlorophyll, and have reduced water use efficiency. Our group is interested in the role of the circadian clock in C4 grasses, in particular, sugarcane.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Using oligoarrays, we have estimated that a third of transcripts from sugarcane leaves are rhythmic in constant environmental conditions. These transcripts are regulated by the circadian clock. This proportion is unusually high. Experiments with other species usually estimate 10% to 20% of circadian clock-controlled transcripts. This suggests that the sugarcane circadian clock is particularly important to this crop, which was bred for high sucrose and high productivity. Transcripts associated with many physiological processes were regulated by the circadian clock in sugarcane leaves, including photosynthesis, sucrose and starch synthesis and degradation, DNA and RNA metabolism, and hormone signalling.

We are now investigating rhythms in plants growing inside in a sugarcane field. This experiment will identify transcripts that are rhythmic driven by the circadian clock and other environmental rhythms. Different organs will be harvested: a source organ (leaf +1), a sink organ specialized in cell division and expansion (internodes 1 and 2), and a sink organ specialized in sucrose accumulation (internode 5).

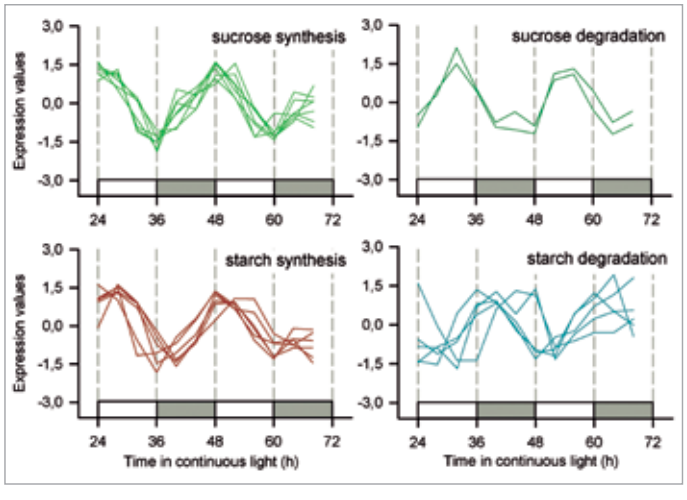

Figure 2. Transcripts associated with sucrose and starch synthesis and degradation are regulated by the sugarcane leaf circadian clock. The transcripts are co-regulated in order to organize different processes in time. During the course of 24 hs, sucrose starts to be synthetized at dawn, followed closed by starch synthesis. Towards the end of the day, sucrose starts to be degraded, followed by starch degradation during the night.

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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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FAPESP Process 2008/56247-6 | Term: May 2009 to Jul 2011

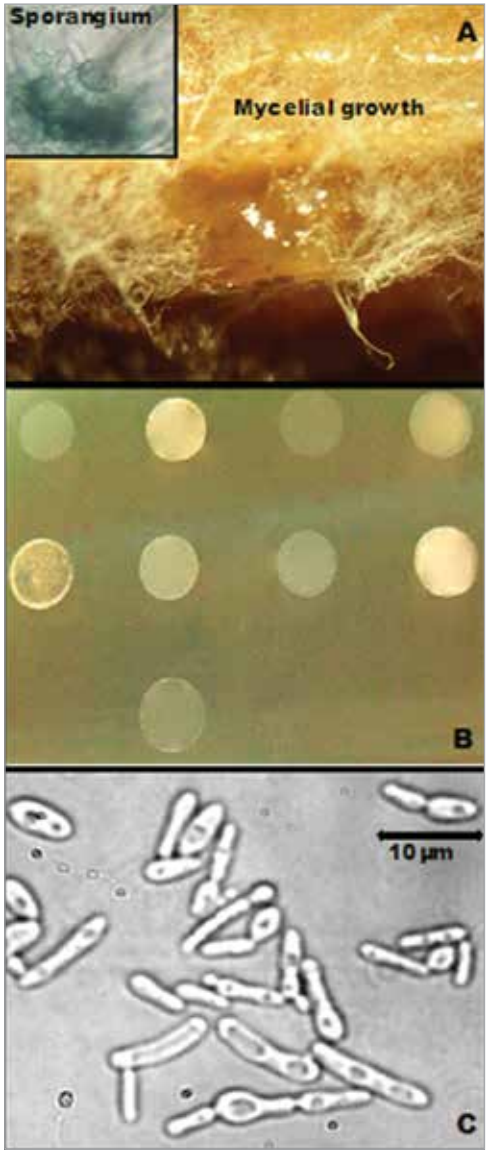

Figure 1. (A) a sporangium (phase contrast microscopy, 1000Í) and the mycelia growth (phase-contrast stereoscopy, 35Í) resulting from the propagation of Aspergillus nidulans *on sugarcane bagasse; (B) Selection of yeasts strains based on growth on plates containing high concentration of both sugar and 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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POPULATION DINAMICS AND INTERACTIONS BETWEEN YEASTS STRAINS IN FERMENTATIONS OF MOLASSES AND HYDROLISATES OF SUGARCANE BAGASSE

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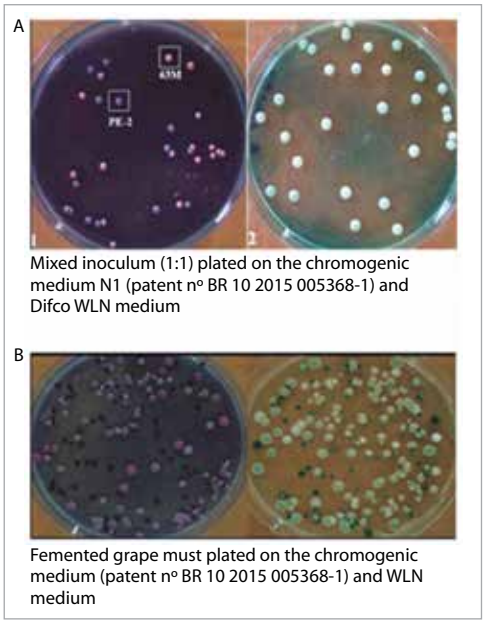
FAPESP Process 2013/15082-2 | Term: May 2014 to Apr 2016

Figure 1. Color of the lignocellulose enriched- fractions obtained by using of modified pre- treatments applied to sugarcane as described by Miranda et al. (2015). SCB is a milled sample of sugarcane bagasse in natura

Thermotolerant yeasts are becoming increasingly important in ethanol production due to the continuing changes in the climate and water scarcity responsible for raises on earth temperature. Ethanol production of first and second generation are complex and long-term processes operating in open reactors with cell reuse. As the cells are exposed for months to successive, oscillatory in aggressive environmental conditions all over the harvest season, the use of thermotolerant yeast is essential. Despite the high capacity of starter cells, compete against other yeast cells present in the environment, process conditions can lead to the arising of modified yeast whose effects on the process must be determined. Genetic variants show chromosomal profiles different from the parental yeasts. Inter-delta sequencing or separation of chromosome DNA bands on electrophoresis gels (CHEF) are tools to identify yeast strains. The techniques base on DNA bands are more time consuming and difficult to perform than the inter-delta technique and this can lead to mistakes in identifying strains. The obtaining of strains more resistant to adverse conditions, the development of a dye-based medium for the monitoring of population dynamics and a less aggressive physical-chemical method to pretreat sugarcane bagasse are intentions of the present study.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

A chromogenic medium (Masiero and Laluce, patent n° BR 10 2015 005368-1) was developed to identify genetic variants originated during fermentation as well as wild yeasts (contaminants) using using inter- delta sequencing. In addition, pre-treatments were improved to obtain less recalcitrant cellulose enriched -fractions from the sugarcane bagasse (Miranda et al., 2015). This medium developed to differentiate colonies of yeasts proved very useful for monitoring of the persistence and permanence of yeasts during fermentation. On the other hand, the highest s biomass yields of the cellulose enriched-fractions resulted from the application of assisted microwave pretreatments to the sugarcane bagasse. The cellulosic fraction PT6 resulted from a microwave pre-treatment in sulfuric acid solution, while cellulosic fraction PT7 resulted from a two-step microwave pre- treatment in sulfuric acid solution followed by a pre- treatment in alkaline solution. The highest yields of glucose (enzymatic assay) were obtained from fractions PT6 and PT7, during extraction with a concentrated sulfuric acid solution (30%, w/v). This indicates the two cellulosic fractions went through acidic degradation during extraction. In addition, the liberation of phenols from lignin increased during incubation of the cellulosic fractions in diluted solution of sulfuric acid (4%-6%, w/v) containing Fe_2Cl_3. Thus, it seems that the lignin attached to the cellulose fractions can be largely removed by extraction with diluted sulfuric acid solutions in the presence of Fe_2Cl_3.



Mixed inoculum (1:1) plated on the chromogenic medium N1 (patent nº BR 10 2015 005368-1) and Difco WLN medium

Femented grape must plated on the chromogenic medium (patent nº BR 10 2015 005368-1) and WLN medium

Figure 2. Chromogenic media for monitoring the yeast population dynamics during grape must (B) and control plates (A) showing purple colonies for the industrial strain (A, part 1) and the mutagenized strain 63M (A, part 2) showing colonies of pink colors . Strain 63M obtained as previously described (Souza et al., 2007)

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PETRODIESEL VS BIODIESEL: A COMPARATIVE STUDY ON THEIR TOXIC EFFECTS IN NILE TILAPIA AND ARMOURED CATFISHES

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FAPESP Process 2008/58032-7 | Term: Feb 2011 to Jan 2013

Figure 1. The potential toxic effects of biodiesel to the aquatic biota have been studied in fish in a project developed by the group of Prof. Dr. Eduardo Alves de Almeida at the São Paulo State University, Campus of São José do Rio Preto

It has been shown that diesel oil is highly toxic to aquatic animals. Much of the biochemical responses activated during diesel oil exposure in fishes involves induction of cytochrome P450, especially 1A isoforms, and glutathione S-transferases. Oxidative stress can be also originated as a result of increased cytochrome P450 reactions and redox-cycling reactions that generate reactive oxygen species (ROS) as by-products. These ROS can oxidize macromolecules such as lipids and DNA, leading to cell death. On the other hand, cells possess antioxidant defenses like the enzymes superoxide-dismutase (SOD), catalase (CAT) and glutathione-peroxidase, which are generally activated during oxidative stress situations. These responses can be accessed in exposed organisms to predict diesel effects. There is increasing interest on the production of biodiesel from plant oils and animal fats, as an alternative for no renewable petroleum-derived diesel oil. Besides biodiesel could be a renewable alternative as fuel, it has been proposed that it is also less deleterious to the environment. However, the toxic effects of biodiesel on aquatic biota is not fully studied. In this project, we aim to investigate how biodiesel can be less deleterious than petroleum diesel to tilapias (*Oreochromis niloticus*), and armoured catfishes (*Pterygoplichthys anisitsi*), through acute toxicity tests (96h) to establish LC100 and LC50 for both species, and the analyses of biochemical biomarker in fishes exposed for 15 and 30 days to sub-LC50 levels of these contaminants. The activities of cytochrome P450 isoforms, GST, CAT, and lipid peroxidation levels will be measured as toxicological parameters to indicate diesel and biodiesel effects.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

LC50 values for B5 and B20 biodiesel were obtained for tilapias (below 0.5 mL/L). Contrarily, the catfish did not die even at concentrations as high as 6 mL/L, indicating this species as very resistant for diesel or biodiesel exposure. On the other hand, both species presented significant alterations in several biochemical parameters. In general, P450 and GST activities were higher as the amount of petrodiesel increased in the mixtures, and no significant alteration was observed for fish exposed to biodiesel. Nevertheless, oxidative stress parameters were altered in all groups of fish exposed to pure diesel oil, mixtures of petrodiesel with biodiesel B5, B20 and even pure biodiesel (B100). These results indicates that, despite its less toxicity compared to petrodiesel, even pure biodiesel can represent a risk to aquatic biota, causing significant alterations in biochemical parameters. The project is just on its first year. We expect more results for the next year.

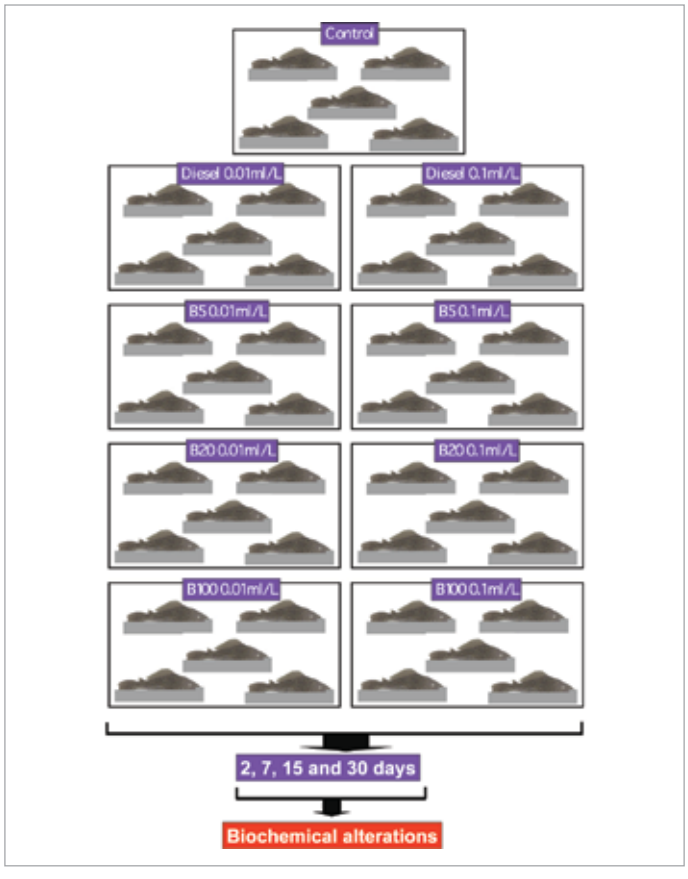

Figure 2. Fish are exposed to pure diesel, biodiesel and biodiesel blends at different concentrations for different periods of time, and then several biochemical parameters are evaluated and the results are compared to an unexposed control group, in order to identify the effects of these compounds to fish, and to evaluate how the addition of biodiesel in petrodiesel can decrease its toxicity to the aquatic organisms

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LIBRARY GENERATION FOR BIOMASS-CONVERSION ENZYMES FROM SOIL METAGENOME

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FAPESP Process 2008/58037-9 | Term: May 2009 to Apr 2014 | Young Investigator

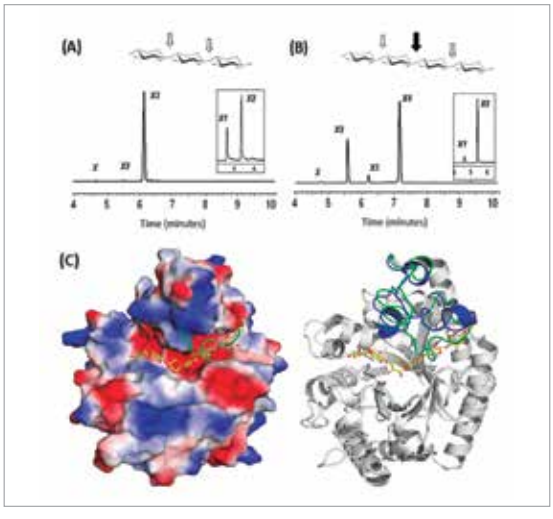

Figure 1. A novel endoxylanase family GH10 (SCXyl) was identified from sugarcane soil metagenome. Along with the description of biochemical characteristics and biotechnological application, SCXyl crystal structure was solved. The ScXyl1 has unusual enzymatic activity against small xylooligosaccharides (A and B) is consistent to the hydrophobic contacts at the +1 subsite and low-binding energies of subsites that are distant from the site of hydrolysis (C). This unusual enzymatic activity is advantageous, because this allows the enzyme to maintain active even in advanced steps of the catalysis, when most of the long xylan chains have been cleaved (Alvarez et al., 2013)

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 microorganisms 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 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.

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 cellulolytic 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.

During the past years, our group has combined high-throughput screening and omics approaches to develop biotechnological routes for production of high-value compounds from plant biomass. We have compiled a collection of enzymes that degrade glycosidic bonds, derived from diverse sources such as soil metagenomes, as fungi, termites, thermophilic prokaryotes and synthetics (artificial) genes. Comprehensive biochemical studies have enabled to assign enzymes to specific biotechnological applications, as well as, molecular dynamics and structural studies have helped to better understand substrate and enzyme interactions at molecular level. Moreover, we have improved fungal systems for protein expression/secretion enabling large-scale production processes.

MAIN PUBLICATIONS

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ISOLATION AND IDENTIFICATION OF microRNAS AND TARGETS IN SUGARCANE

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Figure 1. Cover of the Vol.64 of the Journal of Experimental Botany where our paper entitled "Global analysis of the sugarcane microtranscriptome reveals a unique composition of small RNAs associated with axillary bud outgrowth" was cover

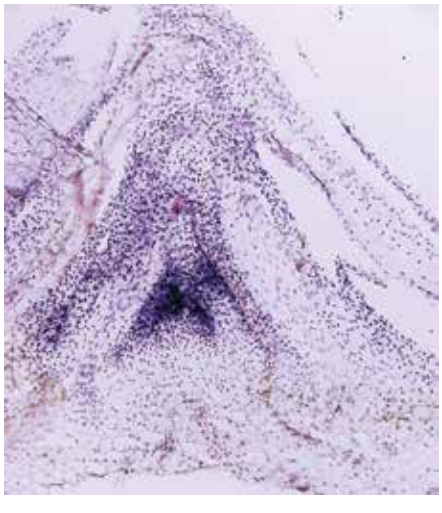

Figure 2. Spatiotemporal expression pattern of the miRNA miR159 in axillary buds of sugarcane. A probe of a 3'-labelled LNA-modified oligonucleotide detecting miR159 was hybridized with longitudinal sections of vegetative buds

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

In our first publication, we computationally identified 19 distinct sugarcane miRNA precursors, of which several are highly similar with their sorghum homologs at both nucleotide and secondary structure levels. The accumulation pattern of mature miRNAs varies in organs/tissues from the commercial sugarcane hybrid as well as in its corresponding founder species *S. officinarum* and *S. spontaneum*. Using sugarcane *MIR827* as a query, we found a novel MIR827 precursor in the sorghum genome. Based on our computational tool, a total of 46 potential targets were identified for the 19 sugarcane miRNAs. Several targets for highly conserved miRNAs are transcription factors that play important roles in plant development. Conversely, target genes of lineage-specific miRNAs seem to play roles in diverse physiological processes, such as *SsCBP1*. *SsCBP1* was experimentally confirmed to be a target for the monocot-specific miR528. Our findings support the notion that the regulation of *SsCBP1* by miR528 is shared at least within graminaceous monocots, and this miRNA-based post-transcriptional regulation evolved exclusively within the monocots lineage after the divergence from eudicots.

In our second publication, we employed sRNA next-generation sequencing as well as computational and gene-expression analysis to identify and quantify sRNAs and their targets in vegetative axillary buds of sugarcane. Computational analysis allowed the identification of 26 conserved miRNA families and two putative novel miRNAs, as well as a number of trans-acting small interfering RNAs. sRNAs associated with transposable elements and protein-encoding genes were similarly represented in both inactive and developing bud libraries. Conversely, sequencing and quantitative reverse transcription-PCR results revealed that specific miRNAs were differentially expressed in developing buds, and some correlated negatively with the expression of their targets at specific stages of axillary bud development. For instance, the expression patterns of miR159 and its target *GAMYB* suggested that they may play roles in regulating abscisic acid-signalling pathways during sugarcane bud outgrowth. Our work reveals, for the first time, differences in the composition and expression profiles of diverse sRNAs and targets between inactive and developing vegetative buds that, together with the endogenous balance of specific hormones, may be important in regulating axillary bud outgrowth.

In collaboration with other groups, we mapped sRNAs in the sugarcane genome and its transposable elements (TEs). The results presented support the conclusion that distinct small RNA-regulated pathways in sugarcane target several lineages of TE elements and maybe several sugarcane loci.

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A SYSTEM FOR LARGE SCALE PRODUCTION OF RECOMBINANT PROTEINS

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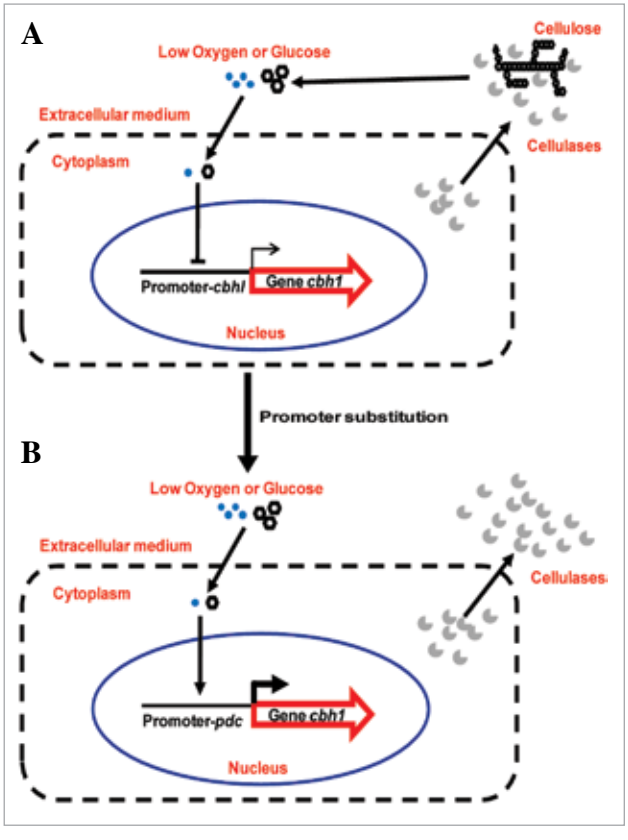

Figure 1. The cellulolytic system of T. reesei *requires induction by cellulose but is repressed by the cellulose degradation product – glucose, or low oxygen tension (A), however, substitution of the promoter (for example, of the cbh1 gene) for the pyruvate decarboxylase (PDC) promoter, which is strongly 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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Chambergo FS, Valencia EY, Ferreira-Júnior JR, Camilo CM, Campana PT. 2012. Conformational stability of recombinant manganese superoxide dismutase from the filamentous fungus *Trichoderma reesei*. Int *J Biol Macromol*. **50(1)**:19-24.

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ANALYSIS OF PROMOTERS FOR THE EXPRESSION OF RECOMBINANT PROTEINS IN TRICHODERMA REESEI

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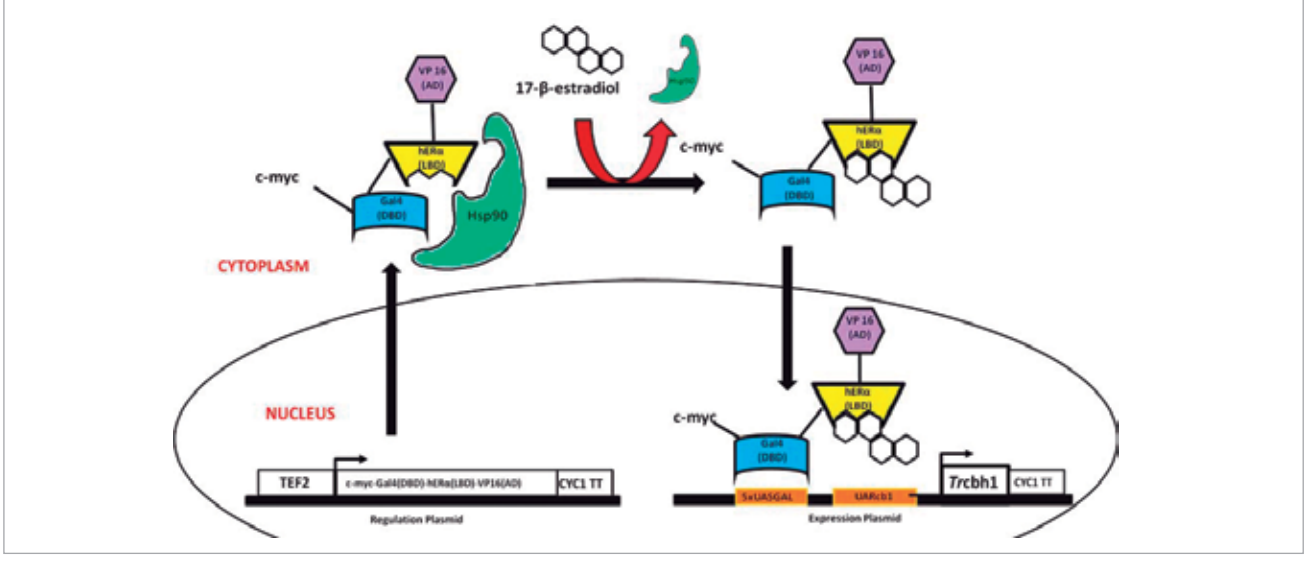

*Figure 1. Induction of the expression of cellobiohydrolase 1 (*cbh1*) gene from* Trichoderma reesei *in the presence of human hormone through the modified* cbh1 *promoter. An estrogen receptor-based gene switch to activate modified Tr*cbh1 *promoter containing five UAS Gal4 motifs from Gal4 gene of* Saccharomyces cerevisiae *was constructed*

The highly efficient secretion machinery of the filamentous fungus *Trichoderma reesei* should be useful for large scale production of homologous or heterologous proteins of industrial interest. We intend to modify a set of metal-induced promoters to allow expression of proteins in large scale using *T. reesei* as host. The design or identification of such promoters would decrease the production costs of enzymes capable of biomass hydrolysis, and that would help the dissemination of biomass use as source of biocombustible.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Our objective is the construction of a system for large scale production of enzymes by means of identification, substitution or modification of *T. reesei* promoters capable of driving a large production and efficient secretion of enzymes involved with the degradation of biomass. The promoter of cellobiohydrolase 1 (*cbh1*) gene of *T. reesei* is induced by cellulose and is strongly repressed by glucose, being commonly used to construct highly efficient heterologous expression vectors. Expression vectors were constructed for *T. reesei* using the following designs: (i) Induction of the expression of *cbh1* and *egl1* genes in the presence of D-sorbitol through the substitution of its native promoter by pyruvate descarboxylase promoter (P*pdc*) and (ii) Induction of the expression of β-galactosidase (*LacZ*) or *cbh1* genes in the presence of metals or human hormone through the modified *cbh1* promoter. *T. reesei* QM9414 mutant strains that express *cbh1* and *egl1* under the control of P*pdc* induced with 200 mM D-sorbitol were analyzed, showing low total protein production (1,3 $g.L^{-1}$ endoglucanase and 0.9 $g.L^{-1}$ celobiohydrolase). Endoglucanase (CMCase) and cellobiohydrolase (Avicelase) activities were 53 IU and 130 IU, respectively, after 24 hours of induction. *Saccharomyces cerevisiae* mutant strains that express *LacZ* were induced by zinc, under the control of *cbh1* promoter modified by inserting Metal Response Elements (MRE) from the *S. cerevisiae zrt1* gene and *S. cerevisiae* strains that express *cbh1* are currently being analyzed for their induction by estradiol, under the control of *cbh1* promoter modified by inserting UASGal elements of GAL4 gene from *S. cerevisiae*. We constructed a set of plasmids with inducible promoters to allow expression of proteins using *T. reesei* as host; however, when using the Ppdc promoter, protein expression is low. Other modified promoters are currently being studied.

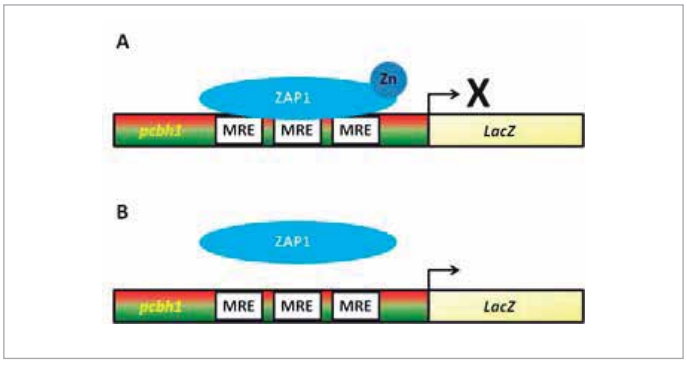

*Figure 2. Induction of the expression of β-galactosidase (*LacZ*) gene in the presence of metals through the modified* cbh1 *promoter from* T. reesei. *An promoter containing three Metal Regulatory Elements (MRE) from zrt1 promoter of* S. cerevisiae, *that express* LacZ *reporter gene under the control of modified Tr*cbh1, *β-galactosidase activity was induced with different Zn concentration. A) High Zn, B) Low Zn*

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MICROBIAL CONSORTIA FOR BIOWASTE MANAGEMENT – LIFE CYCLE ANALYSIS OF NOVEL STRATEGIES OF BIOCONVERSION (MICROWASTE)

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FAPESP Process 2013/50353-7 | Term: Feb 2014 to Jan 2018

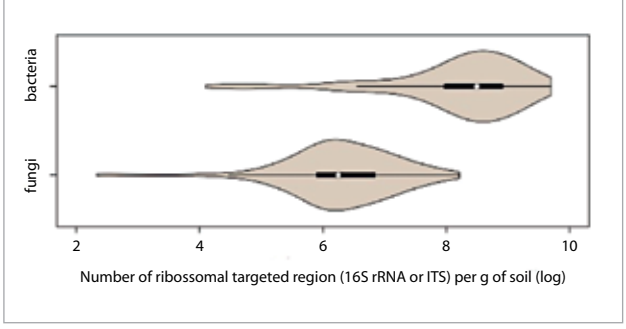

Figure 1. Frequency of quantification values for of Bacteria and Fungi in soils cultivated with sugarcane in distinct locations in Brazil and under differential agricultural managements

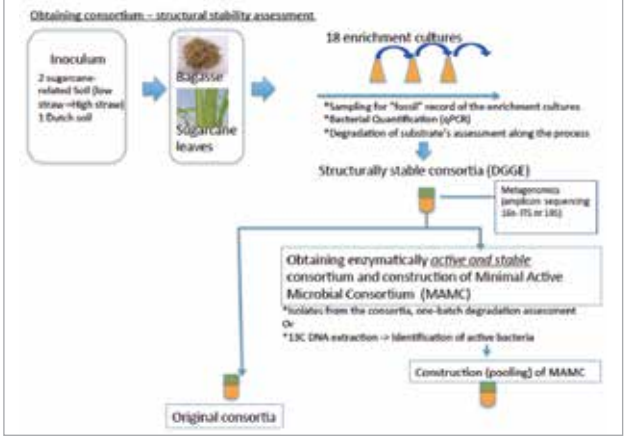

Figure 2. Experimental design for the enrichment and description of microbial consortia involved in the degradation of lignocellulosic material.

Biological waste from agricultural and other sources is a both nuisance and a source of biotechnological opportunities. The project MICROWASTE will develop and foster the understanding of microbial consortia involved in the degradation of the lignocellulosic matter present in agricultural biowaste (sugarcane remains [BR] and maize stalks [NL]). The two research groups have long track records of collaboration and are pioneers in the application of cultivation-independent tools to unravel the soil microbiota in terms of phylogeny and function. Moreover, they also pioneered studies on the interactions between bacteria and fungi in soils and biowaste processing. The current proposal links the activities at the NL partner, which address the key microorganisms of biodegradative consortia and their interactions, as well soil indicators of ecosystem services, with those in BR, which address the soil microbial communities in agricultural and natural ecosystems. Together, we will focus on interactions of key players in microbial consortia and their enzymes (e.g. cellulases, laccases, etc), next to their interactions. We will take a metagenomics approach, which will be combined with network analysis to pinpoint interactive species and key (novel) genes. These approaches will be also used to predict substrate degradability in soils, either with or without an added microbial consortium. Finally, the effect of consortia inoculation on ecosystem services will be assessed. The final outcome of the project will be (1) the availability of a stable microbial consortium which is robust due to its interactivity, with great potential to be applied for monomer production in reactors and in the field, (2) the assessment of the ecological gains of applying biowaste as opposed to the traditional way of burning or disposing it in soil, and (3) the prediction of the effects of biowaste incorporation on the soil processes.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Part I – Tracking microbial interactions in sugarcane fields

Study 1. Deep survey on microbial communities in soils used for sugarcane cultivation under distinct management

This study targets the spatial-temporal dynamics of microbial communities in soils used for sugarcane cultivation along the complete period of cultivation for sugarcane (4 years). We found a great support to develop this approach in the Brazilian Bioethanol Science and Technology Laboratory (CTBE). The results obtained in this first year are related with the quantification of bacteria and fungi in samples collected in the first and second years of cultivation. Data revealed that bacteria are more abundant than fungi in most of samples, and variations in these values were higher for fungi than bacteria. Distinctions among areas will be further addressed, when the complete dataset (with samples from times 0, 1, 2 and 3 years of sugarcane cultivation) are available.

Study 2. Combining the analysis of ribosomal gene sequences with metagenomics in soils samples from sugarcane fields

This step is developed to use data already generated in previous studies (also financed by FAPESP – process 2011/03487-2). The data was collected by sampling soils from sugarcane plantations along 10 distinct regions of the State of São Paulo, and further contrasting the microbial community composition with the environmental data.

At that moment a great dataset was generated, made of 95 soil samples subjected to archaeal and bacterial 16S rRNA gene sequencing, and 9 soils samples (those already showing more dissimilar ribosomal patterns) subjected to a deep sequencing for metagenomics analysis.

The exploration of this dataset was started in 2015, focusing the approaches in the description of microbial groups correlated with the degradation of lignocellulosic materials. It will be correlated the taxonomical analysis performed on ribosomal sequences, with a functional approach, obtained on the basis of the assessment of the metagenomes available from the targeted areas.

Part II – Hitherto a microbial consortia for efficient degradation of lignocellulosic materials

Here the focus is to obtain well-characterized and stable communities that are able to effectively degrade lignocellulosic material, depicting their interactions and ecological roles during the degradation process will be performed. The methodology includes a combination of cultivation techniques and molecular methods - metagenomics and metatranscriptomics.

The microbial consortium was obtained by sequential culturing of inoculant microbial communities (two sugarcane-related soils from Brazil) enriched with sugarcane residues (Bagasse and straw). Consecutive transfers ensured the stability of the communities. Degradability of the substrate was assessed by a gravimetric method. The next step will include meta-omics exploration of the stable consortia as well as isolation and characterization of strains in order to construct a Minimal Active Consortium (MAC) with high degradability properties.

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SUGARCANE SIGNALING AND REGULATORY NETWORKS

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FAPESP Process 2008/52146-0 | Term: Nov 2008 to Oct 2015 | Thematic Project

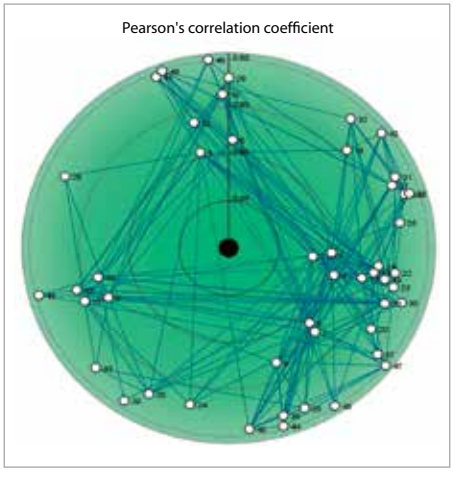

Figure 1. Gene co-expression network with 4CL maize gene. The central dot represents the 4CL gene and the concentric circles show the Person correlation coefficient limits until 0.8 cutoff. Genes with mutual correlation above 0.8 are connected by blue lines

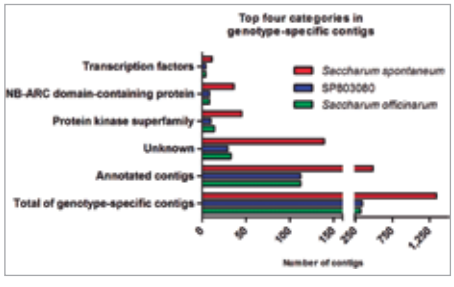

Figure 2. Sugarcane ORFeome top four categories of genotype-specific contigs based on Phytozome annotations. Only contigs from leaf samples in each genotype were considered

We aim to study signaling and regulatory networks in sugarcane and to develop tools for a systems biology 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), continue studies on the Transcriptome, produce transgenics, develop a database and computational 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 the ChIP-HTS 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 oligonucleotide 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.

Aim 1 – Identification of genes associated to sucrose, lignin content and drought tolerance by transcriptome analyses using microarray technique

Aim 2 – Evaluation of gene function through the generation and analysis of transgenic sugarcane plants

Aim 3 – Sugarcane gene promoter sequence and regulatory motif identification by sequencing R570 BAC clones and SP80-3280 genome using 454 platform.

Aim 4 – Development of the SUCEST-FUN Database and computational tools to support all activities of the BIOEN Program.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

In this Thematic Project we have developed a custom oligoarray platform to study the expression of sugarcane genes. The oligoarray contains 21.092 probes, of which 14.554 detect expression from sense transcripts (SS) and 4.137 detect the expression of antisense transcripts (AS). Transcripts with altered expression were identified in samples contrasting for sugar content (2.761 SS, 126 AS), lignin content (420 SS, 17 AS) and drought tolerance (3.379 SS, 243 AS). We also verified that sugarcane has a higher proportion of transcripts with circadian rhythms than other species.

The sugarcane ORFeome was sequenced from two ancestral and one hybrid genotype using a full-length enriched library. We found 38,195 sugarcane-specific transcripts and observed that less than 1.6% of all transcripts were ancestor-specific. Transgenic sugarcane plants were produced overexpressing and/or repressing three candidate genes selected from the transcriptome results. The expression modification of downstream elements was observed with the use of the custom oligoarray. Sugarcane putative promoters from candidate genes were identified by high-throughput sequencing using the Roche 454 platform and by ChIPSeq experiments using an antibody against the RNA polymerase II.

We started to study the sugarcane development and maturation in the field in two different seasons using a System Biology approach. Physiological, morphological and biochemical measurements were collected in four different developmental stages. Samples from four different tissues in four different developmental stages were collected for transcriptome, proteome and metabolome analysis which are being conducted.

During the project, the SUCEST-FUN Regulatory Network Database (CaneRegNet), which contains data and tools of interest for sugarcane functional genomicists and molecular breeders, was developed. The CaneRegNet database assembles different sugarcane databases such as the Sugarcane Expressed Sequence Tags Genome Project, the SUCAST and the SUCAMET Catalogues, which include expression data and the GRASSIUS database. The tools available in the database are: Cane Genome, Cane Transcriptome, Cane Gene Expression, Cane Physiology, Functional Annotation, Cane Transgenic Plant, Generic Tools and Publication. We have started the sugarcane genome sequencing in this project and we have already sequenced sugarcane BACs and shotgun reads using Roche 454 sequencing platform, pair-end and mate-pair libraries using Illumina HiSeq and long reads using Moleculo Illumina.

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DEVELOPMENT OF AN ALGORITHM FOR THE ASSEMBLY OF THE SUGARCANE POLYPLOID GENOME

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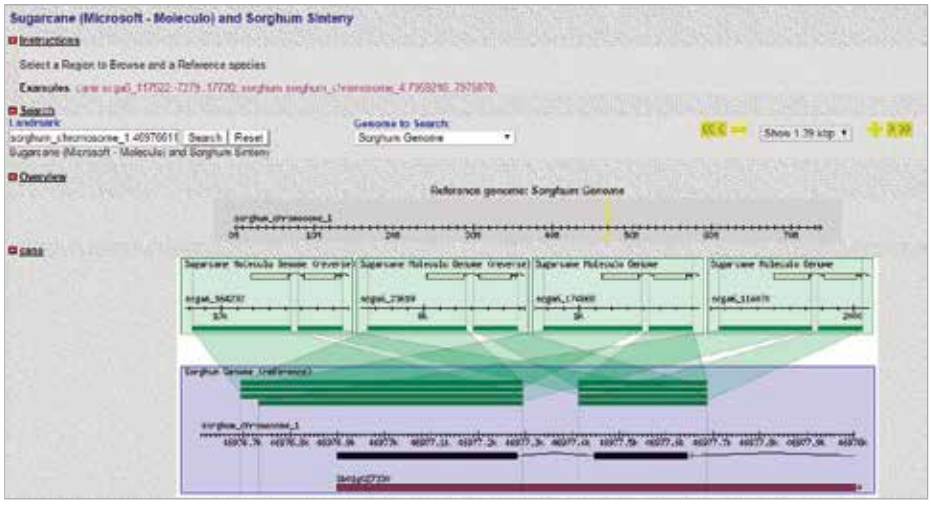

Figure 1. Sorghum and Sugarcane sinteny for one selected sorghum gene showing multiple sugarcane contigs

The challenges in sequencing the sugarcane genome relies in the assembly and analysis of a highly complex genome that is polyploid and aneuploid, with a complete set of homeologous genes predicted to range from 10 to 12 copies (alleles). The present project aims to improve, through the development of new algorithms, the assembly from shotgun reads of datasets generated by the BIOEN research groups. We intend to combine shotgun sequencing data obtained using 454 and Illumina using different protocols to assemble a reference genome for cultivar SP80-3280. To accomplish assembly the group will develop algorithms to enable the recognition of multiple copies of any given region of the reference genome. The group will perform comparative analysis with other genomes, especially sorghum that is very synthenic to sugarcane, to evaluate the quality of the assembled sequence. Existing data on the sugarcane transcriptome will be integrated to validate gene models and infer gene function. Microsoft will develop algorithms for assembly of the sugarcane polyploid genome to strengthen allele identification and improve assembly. The results will contribute to the advancement of knowledge in plant genomics, establishment of computational infrastructure and the formation of highly qualified human resources in bioinformatics. In the long term, we expect to create computational biology tools to resolve polyploid genomes first with the sugarcane genomics but that may also be applied to other cultivated polyploid crops.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Several sequencing platforms (Roche 454; Illumina mate-pair, paired-end and Moleculo) with different characteristics were used to obtain sequences from the sugarcane genome. Approximately 98% of the CEGMA genes were identified in our assembly. Single-copy sorghum genes were aligned to the assembly and the identified copy number varied between one and 15. This shows that it was possible to separate the sugarcane allelic copies in the assembly obtained. Using a custom gene prediction pipeline it was possible to identify approximately 375,000 genes. Orthologues comparisons and transcription active regions identification will be performed to characterize the sugarcane genome.

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BIOENERGY & SUSTAINABILITY

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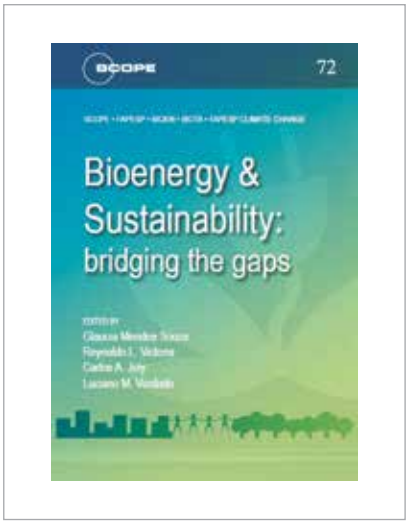

Figure 1. Book cover "Bioenergy & Sustainability: bridging the gaps"

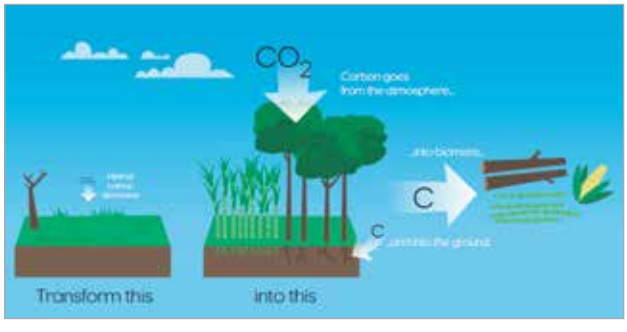

Figure 2. Converting degraded into productive land using biomass

This project aims to conduct a global evaluation of bioenergy sustainability, including advantages, drawbacks, and the criteria to analyze its social, economic and environmental impacts. A *Synthesis of Knowledge* volume and a *Policy Brief,* under the aegis of SCOPE (Scientific Committee on Problems of the Environment), were written with solution-oriented recommendations for public policies on the sustainable expansion of bioenergy.

Technologies that lead to less pollution, lower energy consumption and decreased greenhouse gas emission for bioenergy production and use were evaluated regarding their economic feasibility, the industry capacity for their implementation in scale and the short and long-term impacts on the environment, human health and generation of wealth.

A Scientific Advisory Committee defined both the scope of the synthesis volume and the experts to contribute to the assessment. This committee first met in São Paulo on February 27th and 28th, 2013, at FAPESP, during the *Scoping Meeting*, which had as goal the definition of background themes, authors and reviewers. The *Scoping Meeting* was preceded by an international workshop organized by FAPESP's Programs BIOEN, BIOTA and RPGCC, in which we gathered the broader community's recommendations for bioenergy policies. After producing thirteen background chapters, the roaster of national and international experts met at UNESCO, Paris, on December 2013, for the *Rapid Assessment Process* (RAP) to discuss key findings and recommendations.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Over the past two years, 137 experts from 24 countries and 82 institutions have collaborated to analyze a range of issues related to the sustainability of bioenergy production and use. The resulting report *Bioenergy & Sustainability: Bridging the Gaps* was launched on April 14th, 2015, at FAPESP headquarters in São Paulo, Brazil.

The report references over 2,000 studies, providing a wide-range analysis of the current bioenergy landscape, technologies, production, financing systems and markets, and the potential for sustainable growth of bioenergy use, in parallel with a critical review of its impacts.

The *Bioenergy & Sustainability* report calls attention to the value of bioenergy as an alternative power source and an option to reduce the impact of fossil fuel combustion. It also highlights the opportunities for enhancement of energy security and mitigation of climate change through advanced biomass conversion technologies that would also help to offset the negative environmental impact of deforestation and land degradation due to agriculture and cattle grazing.

Another conclusion of the report is that bioenergy production systems based on sustainable practices can help to offset greenhouse gas emissions resulting from land use changes or loss of biodiversity. These technologies and procedures include combinations of different feedstocks, use of co-products, integration of bioenergy with agriculture, pasture intensification, agro-ecological zoning, landscape-level planning, improving yields, and other land management practices adapted to local conditions.

In addition, the authors affirm that sufficient land is available worldwide for expansion of biomass cultivation, most of this land being located in Latin America and Africa, and that the use of these areas for bioenergy production would not represent a threat to food security and biodiversity under the proper conditions. Moreover, they present evidence that soil improvement technologies, production chain integration and use of bioenergy byproducts in poor rural areas could boost economic performance, enhance food quality and energy access, reduce pollution and create jobs.

To download the full report (open access) please visit: http://bioenfapesp.org/scopebioenergy/index.php/chapters

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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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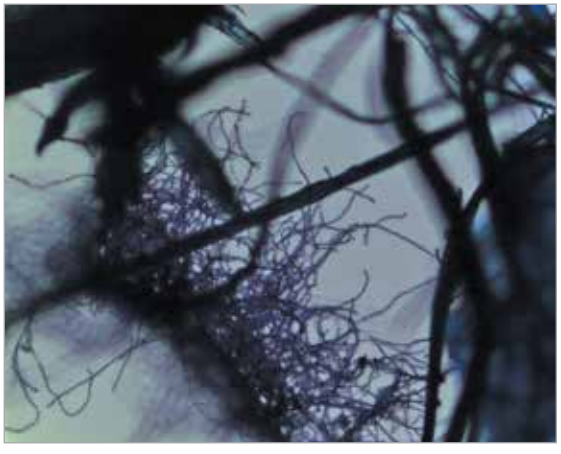
FAPESP Process 2008/56254-2 | Term: Jul 2009 to Jun 2013 | Thematic Project



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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FUNCTIONAL GENOMICS OF PHOTOSYNTHETIC GENES OF SUGARCANE

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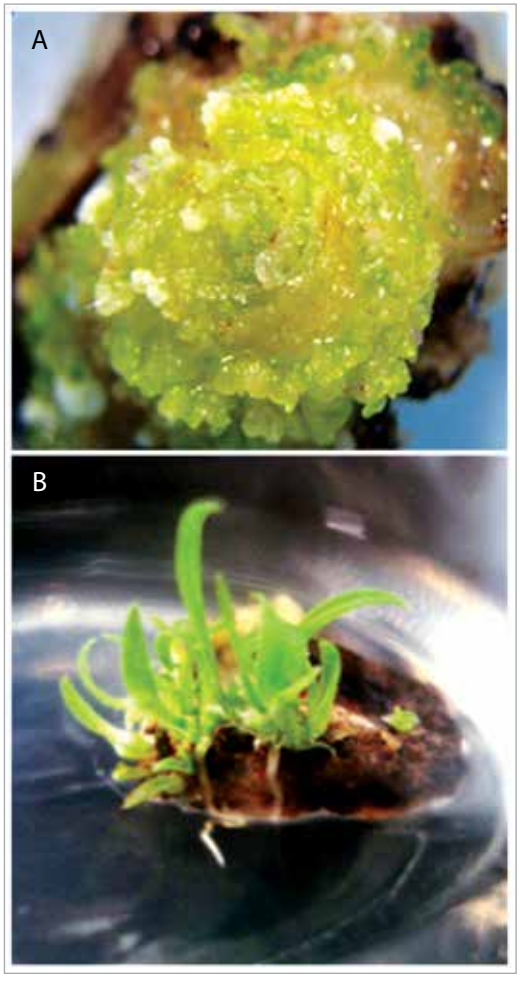

Figure 1. Regeneration of transgenic sugarcane; (A) Somatic embryogenesis from immature leaf-disc; (B) Leaf-disc with transgenic sugarcane

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.

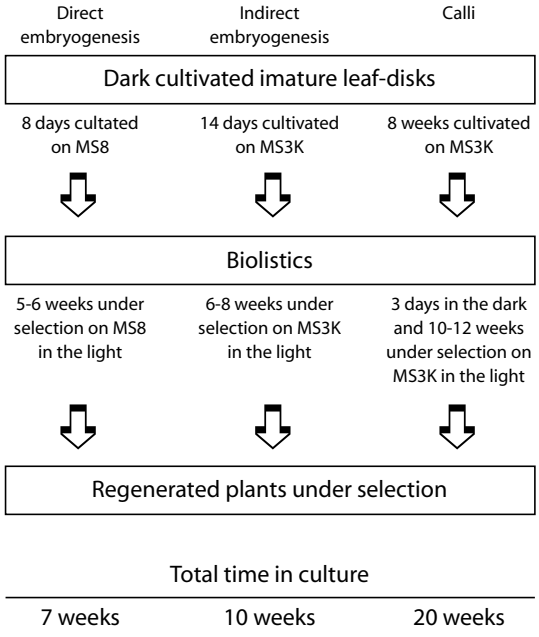

Figure 2. Relative timeframe to generate transgenic sugarcane plants from immature leaf-discs. Somatic embryogenesis was induced through three distinct regeneration processes: direct and indirect embryogenesis and calli

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

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FAPESP Process 2008/57937-6 | Term: May 2009 to Nov 2011

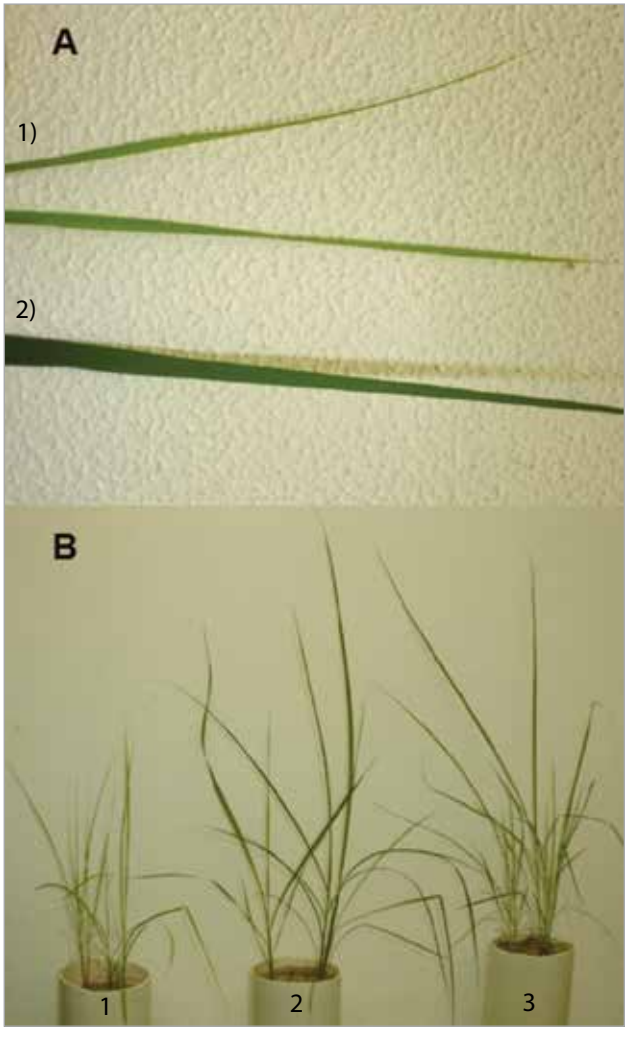


Figure 1. (A) Leaves of sugarcane plantlets treated in absence of combined nitrogen: 1) unfertilized; 2) submitted to organic fertilization. (B) Diferences between leaves lenght of sugarcane plantlets submitted to: 1) no fertilization (control); 2) conventional fertilization and 3) organic fertilization

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

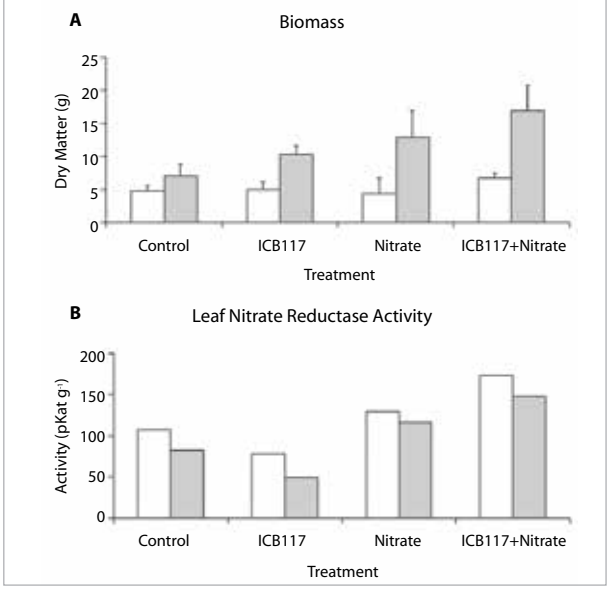
The treatments of sugarcane plantlets, after 60 days of incubation, showed that under organic fertilization, inorganic treatment and in control, respectively:
- a 3.0, 4.7 and 1.7 fold increase in the height of plantlets
- a 15.5, 31.0 and 3.4 fold increase in the fresh weight of plantlets
- a 25.5, 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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FAPESP Process 2008/56255-9 | Term: Nov 2009 to Sep 2014 | Thematic Project
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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.

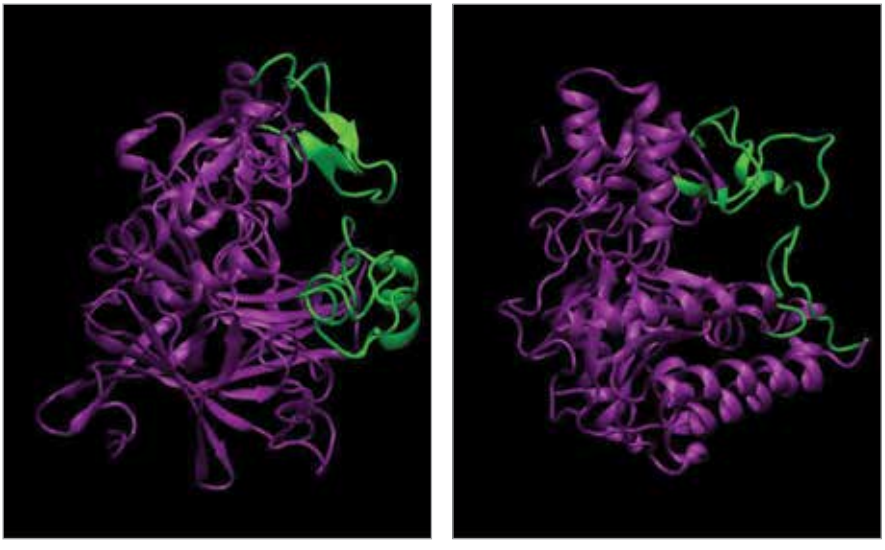

Figure 1. Catalytic core domains of T. reesei *CBH I (Cel7A, left) and CBH II (Cel6A, right). Loops in green highlight tunnel roofs. Tunnel lengths are 50 Å and 20 Å long (out of the plane of the paper) in CBH I (Cel7A) and CBH II (Cel6A), respectivelly*

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.

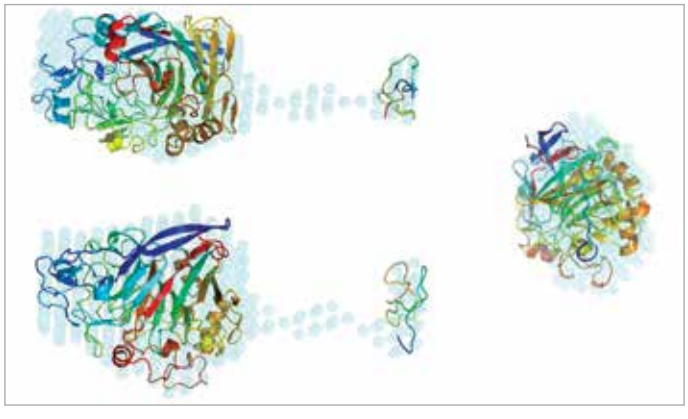

Figure 2. Superposition of small-angle X-ray scattering (SAXS) derived low-resolution envelope of T. harzianum *CBHI with two separate high-resolution structures: of the catalytic domain of CBHI from* T. reesei *and of its cellulose-binding module (CBM). Three orthogonal views are given*

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CENTER FOR BIOLOGICAL AND INDUSTRIAL PROCESSES FOR BIOFUELS (CeProBIO)

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FAPESP Process 2009/52840-7 | Term: Jul 2011 to Jun 2015 | Thematic Project

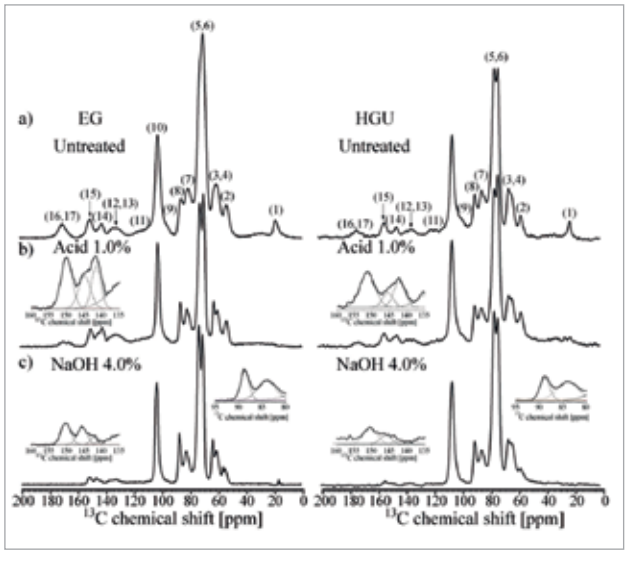

Figure 1. CPMASTOSS NMR spectra of eucalyptus bark samples (Eucalyptus grandis *(EG) and the hybrid,* E. grandis x urophylla *(HGU))* in natura, *after acid and alkaline (NaOH 4%) pretreatment (Lima et al., 2013)*

Center for Biological and Industrial Processes for Biofuels (CeProBIO) is an initiative aimed at articulating the collaborative effort of Brazilian scientific groups already working in various areas of scientific and technological research in the area of biofuels. More specifically, the skills of these groups cover key areas of microbiology, genomics, genetics, molecular and structural biology, enzyme engineering, physical chemistry of the cell wall and bioinformatics, as well as applied disciplines such as mechanical engineering, chemical engineering, environmental engineering and also sustainability, economics and others.

We expect that strong transdisciplinarity and synergies between these skills will enable a quantum leap in the development of technologies of second generation biofuels such as cellulosic ethanol and biodiesel from microalgae. The advances made in this area will materialize in the technologies applicable at a pilot scale and will be based on integrated industrial model, environmentally safe and sustainable, which will serve for study efficiency and process optimization, co-generation of "green" chemicals, waste recovery and optimization of social and economic impacts.

The following eight major projects compose the structural pillars of this proposal: 1. Genetics of plants and microorganisms; 2. Gene discovery and functional genomics to plant cell wall metabolism in cane sugar; 3. Brazilian forests and energy crops for sustainable production of cellulosic, ethanol; 4. Molecular structure of carbohydrates and lignin and the degradation of plant cell walls; 5. Enzymes in bioenergy; 6. Industrial processes for the production of second generation ethanol and co-generation of "green" chemicals; 7. Production of biodiesel from algae in industrial scale and 8. Impacts of biofuel production on water use and carbon emissions.

This proposal will be developed in close collaboration with European proposal SUNLIBB (Sustainable Liquid Biofuels from Biomass), coordinated by Prof. Simon McQueen-Mason, University of York - UK. The SUNLIBB proposal aims at use of genetics, transcriptomics and genomics approaches to improve the quality of biomass for biofuel production; understanding of the polysaccharide composition of the cell wall; cogeneration of products with high added value from biomass; understanding and use of lignin as a raw approach material for the production values; deconstruction of biomass and integrated engineering processes aimed at economic and environmental sustainability in the production of cellulosic ethanol. These aims and objectives are highly complementary with the aims and objectives proposed by CeProBIO and will be performed in a close collaboration between these two centers.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Important advances were achieved in all 8 main projects of the proposal: We´ve conducted molecular genetic mapping of 3 bi-parent populations totalizing over 700 F1 individuals identifying SSR and SNP functional relevant molecular markers, leading to the mapping of important agronomic traits in sugarcane. We also developed and applied the bioinformatics analysis for *de novo* assembly and transcriptome analysis of contrasting sugarcane varieties. Furthermore, novel methodologies for SNPs classification in polyploids and mapping of bi-parent populations with high polyploidy level have been developed and implemented. We built and analyzed full-length enriched cDNA libraries and conducted ORFeome analysis of ancestors (*Saccharum officinarum* and *Saccharum spontaneum*) and one hybrid (SP803280) genotypes. Composition, morphology and processing potential of different sources of Brazilian biomass (including *Panicum maximum*, *Pennisetum purpureum*, *Brachiaria brizantha* and *Eucalyptus*) for sustainable biorenewables and biofuels production were evaluated. Moreover, we determined composition and structure of composition and structure of sugarcane cell wall polysaccharides and studied their implications for second-generation bioethanol production. A number of (hemi)cellulose-active enzymes capable of enhancing lignocellulosic biomass saccharification were identified, produced and tested. We also engineered enzymes and enzyme chimeras with improved biochemical characteristics. Several individual enzymes and enzymatic mixtures were applied to enzymatic hydrolysis of pretreated lignocellulosic biomass. Finally we evaluated impacts of biomass and biofuels production on water usage and carbon emissions, putting forward novel strategies for minimizing environmental impacts and enhancing potential second generation bioethanol production.

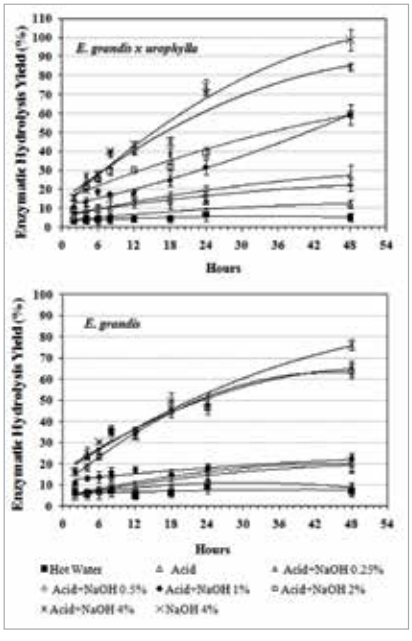

Figure 2. Enzymatic hydrolysis yield obtained for eucalyptus barks after acid and/or alkali treatments along 48 h (Lima et al., 2013)

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FUNCTIONAL CHARACTERIZATION OF THE NEWLY DISCOVERED FAMILY OF MUT9 KINASES IN ARABIDOPSIS THALIANA AND SUGARCANE

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FAPESP Process 2011/50483-2 | Term: Jul 2012 to Jun 2016 | Young Investigator

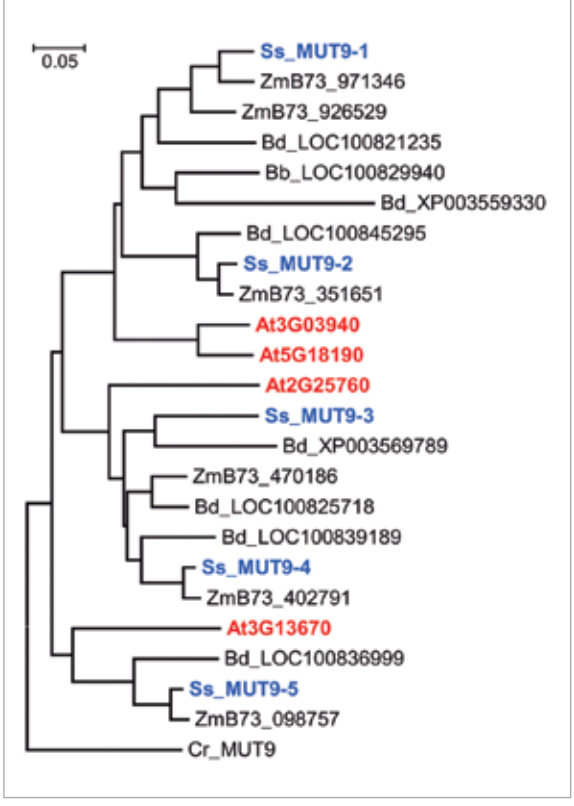

Figure 1. Phylogenetic tree showing the relationship among MUT9 proteins from different plants. Amino acids sequences were aligned by using the ClustalX program, and the tree was drawn with the MEGA 7.0 program. The MUT9 proteins from Arabidopsis and sugarcane are indicated in the tree by red and blue, respectively. MUT9 proteins used to draw the tree are from Arabidopsis thaliana *(At),* Brachypodium distachyon *(Bd),* Chlamydomonas reinhardtii *(Cr),* Saccharum sp *sugarcane cultivar SP80-3280 (Ss) and* Zea mays *B73(Zm)*

The aim of this project is to gain understanding of the role that MUT9 kinases play in the organization and maintenance of gene expression patterns during development, the response to environmental stresses and gene silencing in higher plants. The MUT9 protein was first described as a novel kinase involved in maintaining gene silencing in the green alga *Chlamydomonas reinhardtii*. In this alga the MUT9 kinase is responsible for the phosphorylation of histone H3 at threonine 3 (H3T3ph), which is a covalent modification associated with silent genes and transposons. True orthologs of *MUT9* are conserved in the plant kingdom and have undergone multiple duplications, giving origin to a small gene family with members present in both, mono- and dicotyledonous plants. However, information about the function of these genes in higher plants is almost non-existent. Thus, in order to learn more about *MUT9* genes we are taking advantage of the reverse genetic tools available in the model plant *Arabidopsis thaliana* and the genomic and transcriptomic resources recently developed in sugarcane. In these species, we are studying the spatial and temporal patterns of gene expression of the different *MUT9* homologs. The pathways and genes affected on mutants or lines with disrupted expression of *MUT9* are being investigated by phenotypic and transcriptomic analyses. These studies will be complemented by *in vitro* kinase assays of MUT9 proteins, and analysis of histone phosphorylation and other covalent modifications. With this project, we expect to contribute to the understanding of the molecular mechanisms that eukaryotes use to coordinate their development or to cope with environmental stresses. Preliminary evidence indicates that MUT9 kinases may be involved in regulation development and responses to environmental stresses in higher plants. Thus, the study of the MUT9 kinases could open avenues for the development of novel strategies for manipulation of plant growth and the engineering of stress tolerance in sugarcane and other crop plants.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Searches in genome databases and phylogenetic analysis indicate that the Arabidopsis genome encodes four MUT9 proteins whereas at least five MUT9 orthologs could be identified in sugarcane (Figure 1). These proteins contain a serine/threonine kinase domain and a conserved C-terminal region present exclusively in MUT9 kinases. In Arabidopsis, reverse transcription-PCR (RT-PCR) and promoter fusions with the gene reporter GUS indicate that the four MUT9 genes are expressed in the majority of plant organs. Analysis of translation fusions with the fluorescent protein GFP showed that MUT9 proteins localize into the nuclei, sometimes distributed as a discrete foci. We also isolated homozygous T-DNA mutants in the four Arabidopsis MUT9 genes. Analysis of these mutants did not reveal any obvious phenotypes or developmental defects. However, crossing the T-DNA lines in all possible ways revealed a double mutant combination displaying a drastic phenotype. Plants of this double mutant were dwarf in appearance, often produced floral buds with narrow sepals resulting in exposed floral organs. RNA-seq and a candidate gene approach showed that this mutant is defective in the silencing of developmentally regulated genes.

In sugarcane, RT-PCR analysis of *MUT9* genes in different organs and tissues indicates they are downregulated in mature tissues and also during internode development. RT-PCR analysis also showed that the five *MUT9* genes are drastically downregulated during drought stress in sugarcane roots, and to a lesser extent in leaves. Since H3T3 phosphorylation is dependent on MUT9 activity, we also analyzed the distribution of this modification in nuclei isolated from root cells. In sugarcane interphase nuclei, H3T3ph was distributed as small foci that appear to co-localize with condensed chromatin. Strong H3T3ph signals were also found associated with metaphase chromosomes, indicating an association with chromosome condensation during cell division in sugarcane (*Figure 2*). Our initial observations suggest that MUT9 kinases may play a role during cell differentiation and in responses to drought in sugarcane.

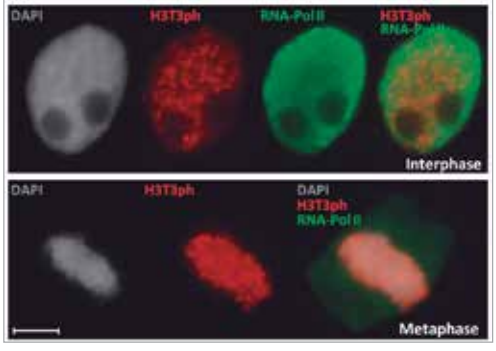

Figure 2. Distribution of H3T3ph and RNA polymerase II (RNA-pol II) in sugarcane root nuclei. H3T3ph (red signals) does not co-localize with actively transcribed regions rich in RNA Polymerase II (green signals). Instead, it appears to be associated with silent chromatin, DAPI densely stained regions (grey nucleus) coincide with H3T3ph brighter foci (red nucleus). Bars = 5 µm. Adapted from Moraes et al. 2015

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BIOENERGY CONTRIBUTION OF LATIN AMERICA, CARIBBEAN AND AFRICA TO THE GSB PROJECT – LACAF-Cane I

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FAPESP Process 2012/00282-3 | Term: Mar 2013 to Feb 2017 | Thematic Project
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Figure 1. Charcoal for cooking in Mozambique: an unfortunate reality

Modern and sustainable bioenergy production can be a sustainable way to meet future demands of energy, particularly for transportation, which remain dependent on limited and environmentally hazardous (i.e. GHG emissions) fossil fuel sources. However, the overall scale of bioenergy production will, in part, depend on the availability of fertile land with appropriate agroecological conditions, thus implying limited trade-offs with food production and biodiversity conservation. In this regard, Latin America and Africa have a significant role to play as, according to some estimates, these regions contain the largest amounts of unexploited arable land that maybe the key to the world sustainable expansion of food and bioenergy. Therefore, project LACAf will focus in these two continents in order to evaluate opportunities and limitations for bioenergy, with emphasis on sugarcane ethanol. Bioelectricity production from sugarcane will also be considered due to its important impacts on rural development and its synergy with ethanol production. Moreover, LACAf intends to be a contribution to the Global Sustainable Bioenergy Project (GSB – http://bioenfapesp.org/gsb/index.php), which was initiated in 2009 by a group of scientists, engineers, and policy experts from universities, government agencies, and the non-profit sector across the globe, with the overall goal of providing guidance with respect to the feasibility and desirability of a sustainable bioenergy-intensive future. The core objective of the GSB Project is to test the hypothesis that it is physically possible for bioenergy to sustainably meet a substantial fraction of future demand for energy services while feeding humanity and meeting other needs from managed lands, preserving wildlife habitat, and maintaining environmental quality.

The LACAf's key questions are:

1 - Why produce bioenergy?
Approach: Regional assessment of bioenergy potential through the integrated analysis of socioeconomic and environmental issues

2 - How much is it possible to produce?
Approach: Land availability and agroecological potential for sugarcane production

3 – How to do it?
Approach: Biofuel production models that may enlarge local gains (socioeconomic) diminish environmental risk and promote investment returns

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The initial activities have involved initiatives to understand and discuss the African countries (more specifically Mozambique and South Africa), trough regionals workshops, meetings and technical missions, in order to answer the following topics: land use & land availability, energy & food security, sugarcane agricultural management, sugar/ethanol production & use, social demands and, sustainability. This first step was essential to consolidate partnerships with local researchers and institutions, to collect & validate data and, to understand clearly the local scenery and demands. Nowadays, the same approach has been conducted in Latin American countries (mainly Guatemala and Colombia). The second step (in course) consider the data analysis and modelling scenery of sugarcane production and expansion aiming to answer the three key questions (Why, How much and, How?). The goal is to produce 20 scientific papers in order to present the results.

Figure 2. Sugarcane plantation to sugar production in Mozambique (Marromeu's Sugarcane Mill): existing know-how and perspectives

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Leal MRVL, Nogueira LAH, Cortez LAB, Cunha MP. 2015. The impacts of ethanol/gasoline equivalence ratio on the sustainability of Brazilian sugarcane ethanol. 21st ISAF International Symposium on Alcohol Fuels. Gwangju, South Korea.

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Nogueira LAH, Souza SP. 2015. Potencial de la caña de azúcar como fuente de energía en América Latina y el Caribe. X Congreso Tecnicaña. Cali, Colombia.

Leal MRVL, Cortez LAB, Leite JGDB. 2015. Agriculture intensification: bridging gaps between food security and biofuel production in Brazil. 2nd International Conference on Global Food Security. Ithaca, New York.

Sheehan A, Sheehan E, Morishige A, Lynd L, Mueller N, Gerber J, Foley J, Martha G, Rocha J, Cortez LAB, West C. 2014. What is the impact of closing the global gap in pasture performance on land use for food and energy in a carbon-constrained world?. Livestock, Climate Change and Food Security Conference. Madrid, Spain.

Leal MRLV, Nogueira, LAH. 2014. The sustainability of sugarcane ethanol: the impacts of the production model. *Chemical Engineering Transactions*. **37**: 835-840.

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Kline K, Leal MRLV. 2014. Food security and biofuels: can policy flexibility reduce food price crises for the poor? Global markets, US maize and Brazil sugar cane cases. 2nd BBEST Brazilian Bioenergy Science and Technology Conference. Campos do Jordão, São Paulo.

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BIOPROSPECTING IN A METAGENOMIC LIBRARY FROM ATLANTIC FOREST SOIL FOR GENES INVOLVED ON THE BIOSYNTHESIS OF BIODEGRADABLE POLYMERS

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FAPESP Process 2010/50473-4 | Term: Jan 2011 to Dec 2013

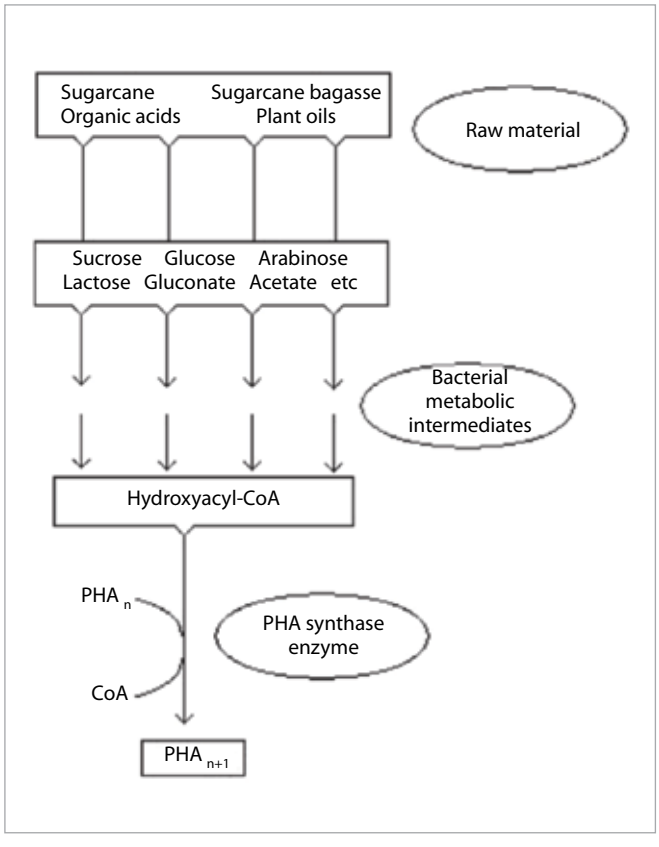

Figure 1. Factors affecting polyhydroxyalkanoate (PHA) production in bacteria

Polyhydroxyalkanoates (PHA) are polyesters accumulated by a wide variety of bacteria from renewable carbon sources and have industrial interest since they are thermoplastics, biodegradable and biocompatible. PHA monomer composition determines its mechanical properties allowing different applications. PHA are composed either by short-chain-lenght (HA_{SCL}) or medium-chain-length monomers (HA_{MCL}) or even HA_{SCL}-co-HA_{MCL} with intermediate properties, more rare due to PHA synthase specificity either to HA_{SCL} or to HA_{MCL}. Three factors are essential to produce PHA: carbon source, bacterial metabolic pathways and the PHA synthase key-enzyme that catalyses R-hydroxyacyl-CoA polymerization to form PHA. Four types of PHA synthase are known depending on their substrate specificity and subunit composition. Types I & II have one peptide unit and types III & IV have two. The project is bioprospecting an Atlantic Forest soil metagenomic library for putative new genes, using phenotypic detection and PCR. Expression of selected genes will be tested on PHA-accumulating *Pseudomonas sp* and *Burkholderia sacchari* mutants impaired on their native PHA synthase. Thus the genes will be expressed on hosts providing diverse metabolic backgrounds to verify if new PHA can be produced. The focus will be HA_{SCL}-co-HA_{MCL} copolymers due their promising properties.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Plasmid DNA from all clones from the metagenomic library was extracted and tested with the six primers described in the literature for the detection of PHA synthase genes. Genomic DNA of *Bacillus megaterium* M.A3.3, *Ralstonia eutropha* and *Pseudomonas aeruginosa* were used as representative controls of each type of PHA synthase. Evaluation of the PCR sensitivity for gene detection in DNA mixtures revealed a detection level of 10-3 ng/µL of the target gene (*Figure 2*). From the metagenomic library, PCR products of the four classes of PHA synthases were found distributed among 80 positive clones. These results are relevant because they suggest that PCR screening, in metagenomic libraries, is more effective than Southern hybridization screening used in reported works found in the literature and also phenotypic detection which fail on finding positive clones. Some randomly selected positive clones were sequenced and confirmed the confidence of the results; presenting a high similarity with PHA synthases of different classes. Next step will evaluate the potential of some of those putative PHA synthases in producing PHA in different hosts. Selected genes will be expressed on two hosts: one with a natural background to accumulate HA_{SCL} and other HA_{MCL}, both affected on their natural PHA synthase. The production of PHA with the target composition will be evaluated in the recombinant strains produced.

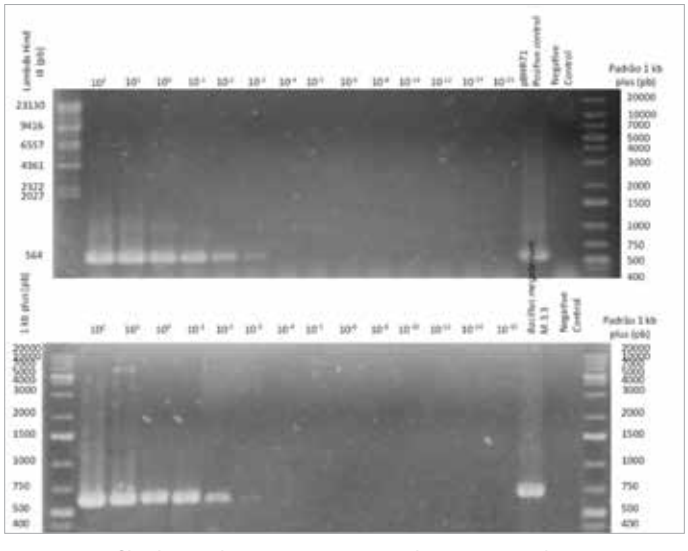

Figure 2. Profile electrophoretic on agarose gel 0.8% (w / v), showing the sensitivity of PCR to detect the minimum concentration (in ng /ul) of the type IV PHA synthase gene of Bacillus megaterium *3.3 MA using the primers in B1F/B1R PCR reaction. The minimum concentration detected by the technique is 0001 ng / ul. At the extremes of the gel can be visualized molecular weight marker 1 kb DNA plus (Fermentas)*

MAIN PUBLICATIONS

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SYSTEMIC ANALYSIS, METABOLIC ENGINEERING AND ECONOMIC EVALUATION OF HEMICELLULOSIC HYDROLYSATE UTILIZATION IN BIOPROCESSES

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FAPESP Process 2010/51989-4 | Term: Jan 2011 to Dec 2013

Figure 1. Biostat B bioreactor performing bacterial cultivation to collect data in the project

Among the sugars of lignocellulose, xylose, present in the hemicellulose, is recovered more easily and in better yields than glucose. Our objective is to increase the efficiency (yield and productivity) of bioprocesses using the biomass hemicellulosic fraction studying the ethalonogenic *Escherichia coli* KO11 and PHA-producing *Burkholderia* species. Polyhydroxyalkanoates (PHA) are polymeric bacterial products that can be used as biodegradable plastics. This research focuses on fundamental understanding of those cellular factors that we consider still the most essential in improvement of xylose utilization by bacteria and of process consolidation aspects to reach the level required in commercial production of biofuels and bioplastics from the hemicellulosic hydrolysates. The following strategies are proposed: (i) construction of genome based *in silico* models of xylose metabolism, (ii) through analysis of those in silico models a metabolic engineering strategy will be proposed to increase yield and productivity of the bioproduct. (ii) ecobiotechnology techniques will be also applied, such as evolutionary engineering to obtain strains with better performance. (iv) high cell density fed-batch cultivations with the best strains will be performed using hemicellulosic hydrolysate as carbon source. (v) From yield and productivity values obtained in this project, a preliminary economic evaluation will be performed to verify the economic potential of this technology.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Multiple copies of the catabolism and transport genes of xylose were introduced in *Escherichia coli* KO11 and *Burkholderia sacchari* LFM 101. Expression of xylA on *E. coli* reduced the rate of xylose consumption and increased final ethanol production by 30% . Results in mixtures of glucose and xylose indicate that the low level of xylAB expression also contributes to the inefficient xylose consumption, in addition to catabolite repression. A faster rate of xylose consumption in the presence of glucose was observed on *B. sacchari* when compared to cultures only in glucose, suggesting that this carbohydrate might have an opposite effect to catabolite repression in this strain (Freire, 2012).

Also to improve aspects on polymer productivity from sugarcane bagasse derivatives, a *B. sacchari* mutant transporting hexoses and pentoses by a non-PTS system uptake system was obtained and presented a released glucose catabolite repression over the pentoses. In mixtures of sugars usually generated on sugarcane bagasse hydrolysate, specific growth rates and specific sugar consumption were 10% and 23% times higher, resulting in a reduced time to exhaust all sugars in the medium. Effects of elimination of PTS components over carbon flux distribution and PHA biosynthesis were evaluated (Lopes et al., 2011 a, *see figure*).

A similar approach was applied to a newly isolated *Bacillus sp*, by deleting the ccpA gene, resulting on a partial release on catabolic repression of glucose over pentoses and a faster consumption of carbohydrates (Lopes et al., 2011b).

Economic assessment of biopolymer production in a sugarcane-based biorefinery context was performed, focusing on a PHA production from xylose in the context of a standard sugar and ethanol plant, cogenerating steam and electrical energy from sugarcane bagasse and agricultural residues. PHA production from xylose, discarded in most mills nowadays, may enable profitability of 2nd generation bioethanol. Productivity varied from 0,28 to 1,11 g/L.h, and PHB price ranged from R$ 4,50 to R$ 9,00/kg. Bioreactor cost was studied in 3 scenarios from US$ 475 to 3.013 thousand and the production capacity was analyzed in ten different scenarios, from 1,000 to 35,000 thousand tons/year. Result reviews offer contribution margin, net operational profit, as well as breakeven point analysis. Recommended is to redirect part of the research efforts from improving PHB yields to process productivity improvement, which turned out to be the key factor to economic feasibility (Raicher, 2011).

MAIN PUBLICATIONS

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Gomez JGC, Méndez BS, Nikel PI, Pettinari MJ, Prieto MA, Silva LF. 2012. Making green polymers even greener: towards sustainable production of PHA. In: *Adv. Appl Biotechnol.* Petre M (editor) p. 41-61, ISBN 978-953-307-820-5.

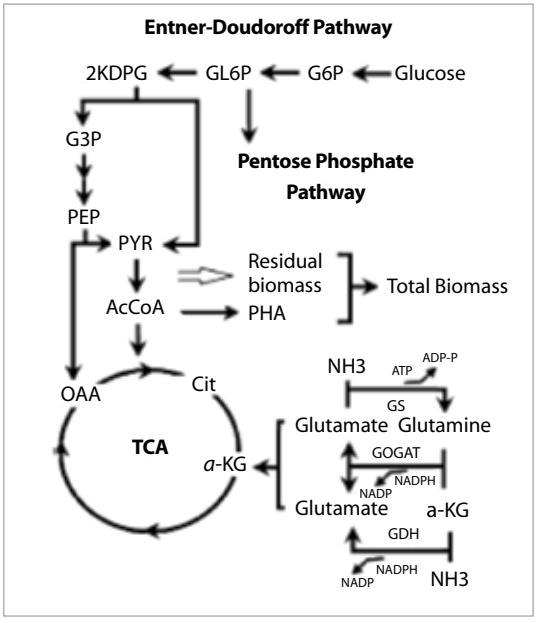

Figure 2. Schematic representation of the polyhydroxybutyrate (PHB) metabolism in B. sacchari. *Glutamate is metabolized to a-KG and then converted to oxaloacetate in the tricarboxylic acid cycle (TCA). Oxaloacetate (OAA) can yield pyruvate (PYR) after decarboxylation and then acetyl-CoA (AcCoA) can be used to produce PHB (Lopes et al., 2011a).*

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TAILOR-MADE BIODEGRADABLE POLYMERS MADE BY BACTERIA FROM BIOMASS

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FAPESP Process 2012/51533-6 | Term: Jul 2013 to Jun 2015 | FAPESP / BE-Basic

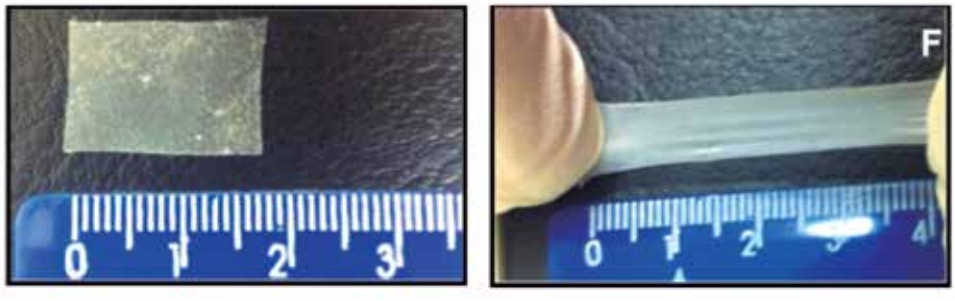

Figure 1. Elasticity of copolymers P(HB-co-20mol%HHx) films produced by recombinant B. sacchari (Mendonça, 2014

The project combined different strategies to produce the biopolymer P(HB-*co*-HHx) controlling monomer composition and contributing to reduce the cost of production. Recent papers and patents have shown that P(HB-*co*-HHx) is a promising biodegradable copolymer of the polyhydroxyalkanoate family (PHA), combining the short-chain (SC) monomer hydroxybutyrate (HB) and the medium-chain (MC) monomer hydroxyhexanoate (HHx), improving properties and applications, when compared with PHA with only SC or MC monomers. Since it can be processed into films, it is interesting to reduce its cost of production to make it a commodity. *Aeromonas spp* naturally produces a copolymer with 5-25 mol% HHx units, however only 10-20% of cell dry weight (CDW) is accumulated as PHA. On the other hand, *Burkholderia sacchari* accumulates up to 80% of CDW as PHB, but incorporates up to 2mol% HHx.

HB fractions are obtained feeding bacteria with a sugar or sugarcane bagasse derivatives, however HHx incorporation depends on feeding with more scarce and expensive medium-chain fatty acids (MCFA). Raw material can represent up to 40% of costs of PHA production thus, is of high interest to find sustainable alternatives with lower cost. It was found that MCFA could efficiently be produced with a mixed microbial fermentation (MMF) technology by anaerobic digestion, with the potential to transform acidified organic waste streams (acetic acid, ethanol etc.) into MCFA mixtures with a hexanoic acid yield of 0.85 mol C/mol C substrate in synthetic medium. MMF was adapted to and optimized for food residues and real feedstocks (e.g. sugarcane bagasse) to generate MCFA-containing streams appropriate to feed bacterial PHA production. This involved setting required effluent standards of MMF for the type and concentration of the fatty acids, or for other disturbing components (N or water content). Operation conditions and appropriate organic waste streams were selected, based on the requirements to feed PHA production. The global objective of the project was to start from a single bacterial species, introduce improvements to produce tailor-made copolymers, controlling monomer composition A relevant issue was to combine and to improve two biological processes (MMF and PHA production) to contribute to sustainably produce an environmentally friendly material starting from low-grade biomass.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

A *Burkholderia sacchari* recombinant was constructed with the ability to incorporate up to 22 mol% 3-hydroxyhexanoate (HHx) units in a copolymer containing also 3-hydroxybutyrate (HB), a considerable increase considering the value of 1.6 mol% HHx incorporated by the wild type strain. The composition of the copolymer P(HB-*co*-HHx) was modulated depending on the combination of the genes expressed and the glucose/hexanoic acid concentration provided. 3-hydroxyvalerate (HV) and 3-hydroxyheptanoate (HHp) monomers could be also incorporated under specific conditions.

Simultaneously to strain improvement, ChainCraft B.V. (Amsterdam, The Netherlands) applied mixed microbial fermentation technology (MMF) to convert residues and biomass into mixtures of medium-chain fatty acids (MCFA) appropriate to be supplied to the recombinants resulting polymers with variable MC monomer compositions, thus with variable properties needed to different uses. Regarding to metabolic fluxes analysis (MFA), based on data from bioreactor experiments with *B. sacchari*, harboring the phaPCJ operon from a locally isolated *Aeromonas sp*, a metabolic model was proposed representing the experimental data obtained. The model predicted that the strain presented a cyclic Entner-Doudoroff pathway for PHA synthesis in the intervals tested. Despite the considerable improvements obtained in converting hexanoic acid to HHx, the strain is operating at 50% of the maximum theoretical value, thus further improvements are currently under study, based on MFA. Analysis of the properties of the copolymers showed that glass transition temperature (*Tg*) and melting temperature (*Tm*) depend on HHx content. According to the properties exhibited by these copolymers, application on flexible porous films or controlled release systems for medicine and dentistry are suggested.

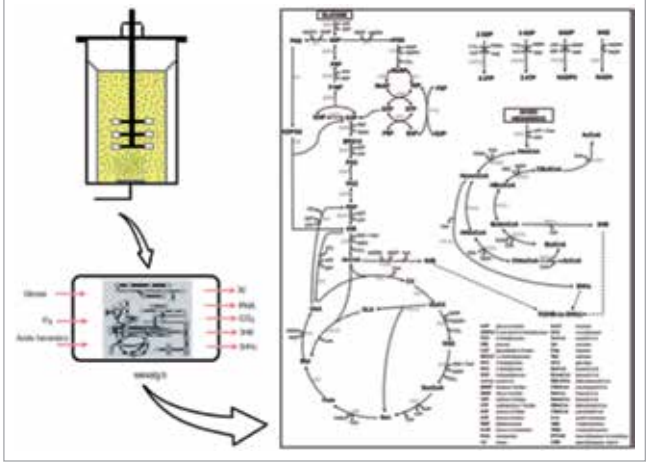

Figure 2. Metabolic Flux Analysis: metabolic model proposed to B. sacchari (Mendonça, 2014)

MAIN PUBLICATIONS

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ANALYSES OF DROUGHT TOLERANCE IN SUGARCANE USING TRANSCRIPTOMICS AND METABOLOMICS

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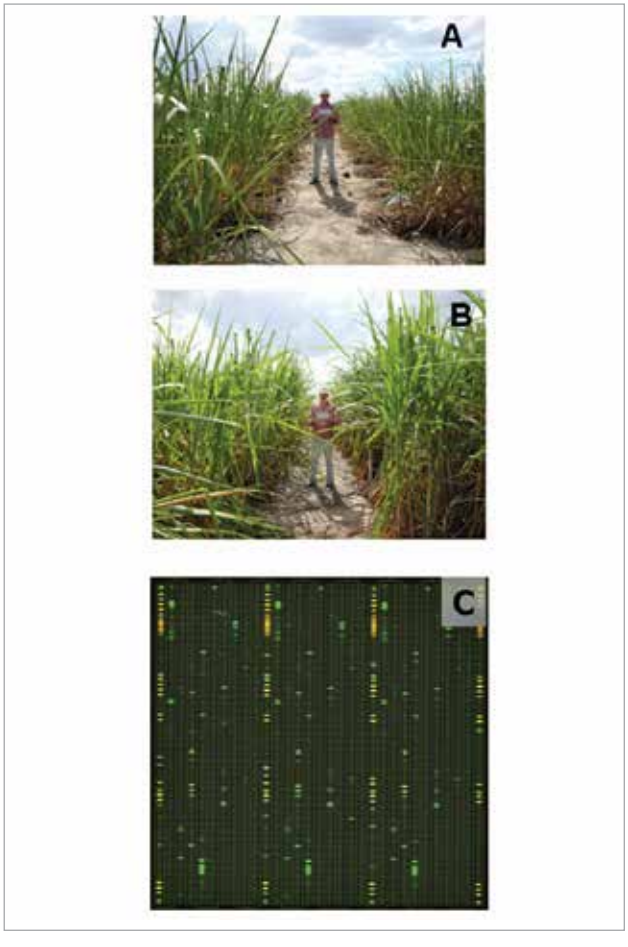
FAPESP Process 2008/56119-8 | Term: Feb 2009 to Jul 2012



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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Papini-Terzi FS, Rocha FR, Vêncio RZ, Felix JM, Branco DS, Waclawovsky AJ, Del Bem LE, Lembke CG, Costa MD, Nishiyama MY Jr, Vicentini R, Vincentz MG, Ulian EC, Menossi M, Souza GM. 2009. Sugarcane genes associated with sucrose content. *BMC Genomics*. **10**:Article Number:120.

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MOLECULAR STRATEGIES INVOLVED IN PLANT-INSECT INTERACTIONS

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FAPESP Process 2008/52067-3 | Term: Mar 2009 to Jul 2014 | Thematic Project
co-PI: Daniel Scherer de Moura

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

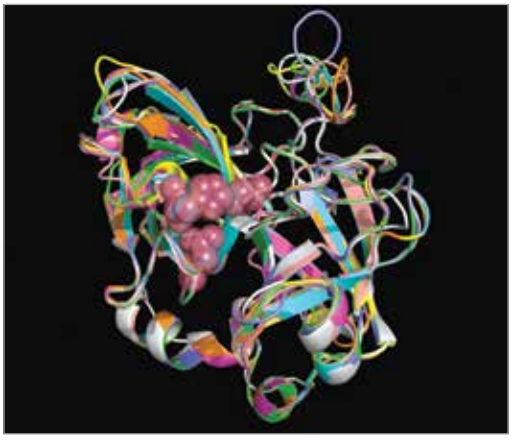

Figure 2. Structural alignment of eight chymotrypsins paralogues modeled by similarity. In detail, the position of the catalytic site

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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DECIPHERING THE MOLECULAR MECHANISMS INVOLVED IN THE LOCALIZATION OF ORGANELLAR PROTEINS AS WELL AS THE COMPLEX PLANT-INSECT-PATHOGEN INTERACTIONS

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FAPESP Process 2014/50275-9 | Term: Feb 2015 to Jan 2020 | Thematic Project

*Figure 1. The Plant Journal front page with the "*FtsH2 and FtsH5: two homologous subunits use different integration mechanisms leading to the same thylakoid multimeric complex*" manuscript from Rodrigues et al. 2011. The Figure illustrates the two-step processing mechanism of the FtsH2 and FtsH5 subunits and their integration into thylakoid membrane by Tat and Sec pathways, respectively*

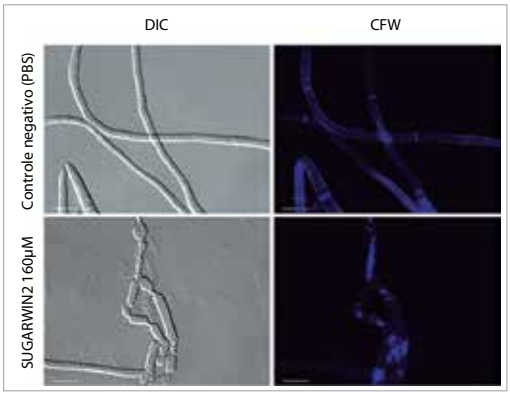

Figure 2. Effects of recombinant sugarcane wound-inducible protein 2 (HisSUGARWIN2) on the hyphal morphology of Colletotrichum falcatum. Calcofluor assay on C. falcatum. C. falcatum germlings were grown in the absence of HisSUGARWIN2 for 16 h of exposure to phosphatebuffered saline (PBS) at 25uC (control) or in the presence of 160 mM HisSUGARWIN2 for 16 h at 25uC. CFW = Calcofluor White. The bars represent 10 mm. (Franco et al., 2014)

Over the last two decades our laboratory has been involved with research and higher education training on the understanding of the molecular mechanisms related to protein subcellular localization, using the model plant *Arabidopsis thaliana*, as well as on the characterization of the complex plant-insect-pathogen interactions.

Regarding the intracellular protein trafficking, we have been working on the identification and characterization of the protein import specificity into their respective subcellular compartments, mainly mitochondria and chloroplasts. We have been able to identify and characterize dual-targeted (DT) proteins and also describe the mechanisms of membrane proteins insertion at organelles (*Figure 1*). Furthermore, we are also working on the identification of auxiliary protein factors acting as regulatory mechanism of organellar membrane proteins.

Our interest on plant-insect-pathogen interactions is related to the bioenergy crop, sugarcane, mainly on the identification and characterization of plant genes involved with the sugarcane borer (*Diatraea saccharalis*) response as well as with opportunistic fungi associated with this interaction. We are also particularly interested on the insect adaptation mechanisms, which allow them to overcome the plant defense barriers, as the peptidase inhibitors (PIs). PIs are essential proteins involved in plant resistance to herbivorous insects. In turn, many insect species are able to escape from the negative effects of these molecules by different mechanisms. One of our main questions is to understand the arms race between plants and herbivore insects in terms of evolutionary mechanisms leading to insect adaptation or successful barriers to prevent insect damage.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

In the intracellular protein trafficking study, we have been able to identify dual-targeted (DT) proteins and also to describe for the first time a post transcriptional mechanisms in plants based on an alternative translation initiation, capable of determining the differential localization of THI1 protein in Arabidopsis. In addition, we also performed evolutionary studies with respect to DT proteins and its conservation among plants. Our results showed that the dual-targeted condition observed in the aminoacyl-transfer RNA (tRNA) synthetases (aaRS) is a gain-of-function derived from gene duplication events and that this condition is maintained among other plants. Regarding the mechanism of integration organellar of some proteins we showed that even high homologues proteins, as the FtsH type A and type B subunits, could use distinct integration mechanisms to lead to the same thylakoid multimeric complex (*Figure 1*).

In the plant-insect-pathogen study we characterized an antifungal protein, SUGARWIN, induced after the sugarcane borer (D. saccharalis) damage and pathogen infection. This protein affects the *Colletotrichum falcatum* (*Figure 2*) and *Fusarium verticilioides* morphology leading to the fungal cell death by apoptosis. This relationship between insect and fungal was further investigated, showing that the presence of both fungal species attract and positively influence *D. saccharalis* feeding, at the same time that the presence of this insect is favorable for the fungal proliferation in sugarcane.

In our studies with peptidase inhibitors, we observed that different Lepidoptera species have different susceptibility levels to these molecules, particularly, we observed that *D. sacharalis* (Crambidae) is more susceptibility to soybean PIs than *S. frugiperda* (Noctuidae). In order to understand this variable response, we developed several experimental and in silico studies, which showed that *S. frugiperda* larvae present a more variable set of trypsin enzymes and a general up regulation in their chymotrypsin and trypsin genes after the inhibitor challenge, while *D. sacharalis* larvae present less variable trypsin genes and absence of expression modulation in their chymotrypsin and trypsin genes in the same conditions. An evolutionary study showed that this could be result of an adaptation of Noctuidae species, originated from an expansion of the trypsin gene family.

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

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FAPESP Process 2008/52074-0 | Term: May 2009 to Jan 2015 | Thematic Project
co-PIs: Claudia Barros Monteiro Vitorello, João Paulo Funio Whitaker Kitajima, Maria Magdalena Rossi

Figure 1

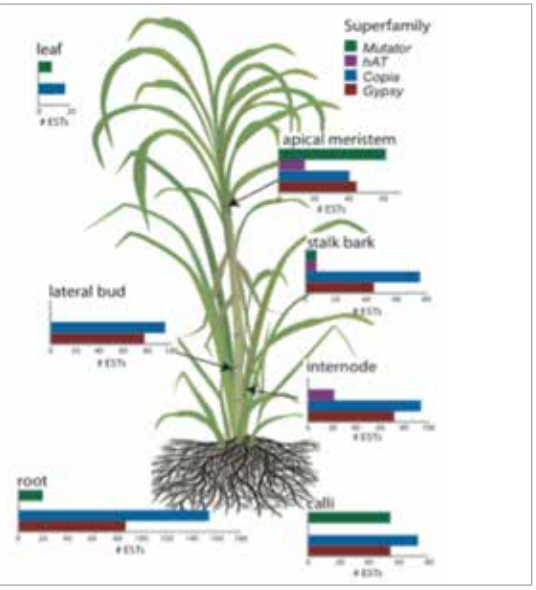


Figure 2

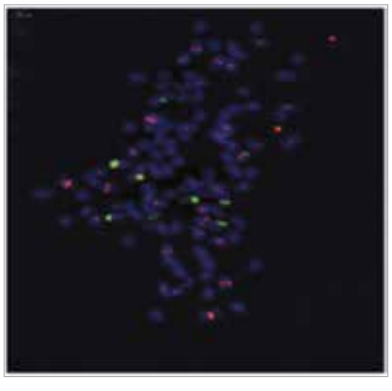
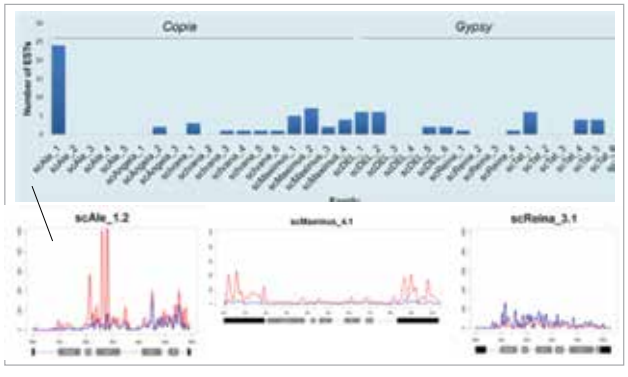


Figure 3



Sugarcane is the major feedstock used in Brazil for biofuel production and is 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. Increased competitiveness in the Brazilian Sugarcane Industry is anticipated pending the increase in total biomass yield and the avoidance of the use of new land for farming. In addition, efficient use of bagasse as biomass is vital to the net yield for the entire production chain. This project aims to generate the 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 based 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 polyploid genome is the result of a recent hybrid cross between *Saccharum officinarum* and *S. spontaneum* Available resources are an EST collection generated by SUCEST, array hybridization profiles generated from SUCESTFUN and a collection R570 BAC clones. The BIOEN program also constructed an SP80-3280 BAC library which will be screened for homologous R570 BAC sequenced locus in order to address allelic variation not only in coding regions but also within regulatory sequences. Transposable elements (TEs) mapping onto these sequenced BACs, array based expression profiles and insertion polymorphism study will provide information on their association with 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 400 sugarcane BAC clones have been sequenced and 280 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 whose broad expression pattern is 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 correlates with higher expression level suggesting that this element is potentially active. From the BAC collection, a BAC containing the rDNA cluster and another centromeric sequences were used in FISH experiments *Figure 2*. Individual families had distinct transcript and sRNA mapping profiles (*Figure 3*), suggesting that they are differentially expressed and regulated. The *Ale*1 family was particularly unusual in that it had 'body-gene'-like sRNA pattern, it is the most transcriptonally active LTR-RT in sugarcane and is concentrated in euchromatic regions. Overall, our results support the TEs could impact the genome in different ways at the family levels.

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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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FAPESP Process 2008/52071-0 | Term: Oct 2009 to Sep 2014 | Thematic Project

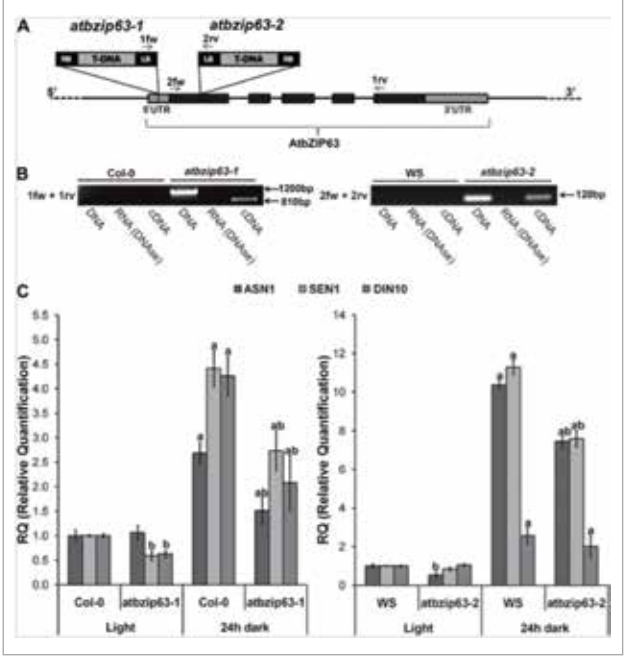

Figure 1. Putative AtbZIP63 target genes. The expression of ASN1 (At3g47340) and SEN1 (At4g35770) is misregulated in two AtbZIP63 T-DNA insertion mutants after dark-induced energy starvation. A, Schematic representation of T-DNA insertion sites in atbzip63-1 and atbzip63-2 mutants. LB, T-DNA left border; RB, T-DNA right border. Primers used to locate T-DNA insertion and detect chimeric transcripts are indicated by the arrows. B, PCR amplification from genomic DNA of the T-DNA insertion region and RT-PCR after DNase treatment showing chimeric transcripts between AtbZIP63 and T-DNA in both atbzip63-1 and atbzip63-2 mutants. Size differences between amplification products from genomic DNA (1,200 bp) and cDNA (810 bp) in atbzip63-1 are due to introns that are absent in spliced AtbZIP63 mRNA. C, ASN1, SEN1, and DIN10 transcript accumulation in atbzip63-1 (Col-0 ecotype) and atbzip63-2 (Ws ecotype) 6-d-old seedlings after 24 h of dark treatment. Significant differences related to seedlings of the same genotype without dark treatment (light) are represented by the letter a (n = 3; P , 0.05) and those between equally treated mutants and their respective wild-type genotype are represented by the letter b (n = 3; P , 0.05). (adapted from Matiolli and al., 2011)

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.

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, by interacting with the circadian clock, and 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. The present proposal aims at unraveling new mechanistic aspects of sugar signal transduction in plants and more broadly at how energetic homeostasis is controlled. 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.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The *Arabidopsis thaliana* (*A. thaliana*) Transcriptional Regulatory Factor (TF) of the basic leucine type AtbZIP63 is a key regulatory node that integrates energetic status, abiotic and biotic signals to adjust growth and development in phase with the diurnal cycle. Comparative RNA profiles analysis and Chromatin Immuno Precipitation approaches revealed that AtbZIP63 interacts with the circadian clock to control starch degradation and is a key intermediate of SNF1 Related kinases 1(SnRK1)-mediates energetic responses (*Figure 1*). Deregulated starch usage at night in atbzip63 mutant lines resulted in severe growth defects involving reduction of cell wall expansion. We are in the process of unraveling new aspects of the regulatory networks in which AtbZIP63 is involved and we mainly are focusing on its role in mediating the interaction between the two functionally antagonist and evolutionary conserved pathways that control growth in response to the available energy: the Target of Rapamycin (TOR)-related pathway and the SnRK1-related pathway.

We found that the control of AtbZIP63 expression is under a complex set of regulatory mechanisms including, transcriptional, mRNA decay control and protein degradation. The stress-related hormone Abscissic Acid (ABA) and Glucose interact to promote AtbZIP63 mRNA degradation. The underlying mechanisms are being investigated. This data prompted us to analyze at genomic scale the extent of mRNA decay regulation mediated by ABA and/orGlucose. We found that ABA possibly negatively feed-back regulates its own signaling pathway probably as a way to reset the signalization by promoting destabilization of mRNA of ABA receptors and ABA-activated TFs. The mechanisms involved are being investigated.

We obtained clear evidences for the existence of a mannose-specific signaling pathway and the details of the signaling process are being revealed including the identification of a potential Mannose receptor. Our working hypothesis is that Mannose represents a Cell wall-derived Damage Associated Molecular Pattern.

We identified a de novo originated Arabidopsis thaliana gene called QQS which is involved in the control of starch metabolism. QQS is prone to epigenetic switches that impact its expression in a manner independent of genetic variation. This gene represents a new tool to obtain new insights into the role of epigenetic variation in adaptation/evolution and how epialleles arise (*Figure 2*). We found more recently that the epigenetic state of QQS and developmental signal interacts to define the expression pattern of this gene during development and obtained evidences that the demethylase ROS1 is also involved. Remarkably, QQS is specifically demethylated in the male germ line cells and we propose that this event is important step in QQS evolution.

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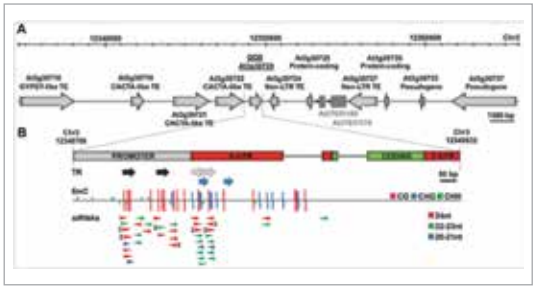

Figure 2. QQS is embedded in a repeat-rich region. (A) Genomic structure of the QQS locus (30 kb window) in the Col-0 accession. Dark grey boxes represent TE sequences. (B) Magnified view of the QQS gene and upstream sequences, showing tandem repeats (TR), methylation of cytosine residues (5 mC) at the three types of sites (CG, CHG and CHH, H = A, T or C) and locus-specific sense and antisense siRNAs (numbers referring to copy number). (adapted from Siveira et al., 2013)

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DISCOVERY AND DEVELOPMENT OF NEW ANTIBACTERIAL AGENTS AS AGROCHEMICALS FOR SUGARCANE CULTURES: BIOLOGICAL SCREENING, MEDICINAL CHEMISTRY AND STRUCTURAL BIOLOGY

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FAPESP Process 2011/08042-9 | Term: Oct 2011 to Sep 2015 | Young Investigator

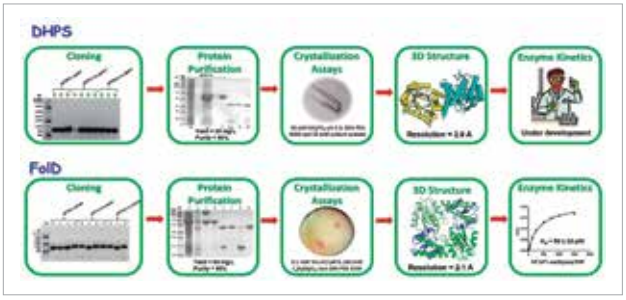

Figure 1. Structural molecular biology strategies toward the determination of the 3D structural of key molecular targets from X. albilineans

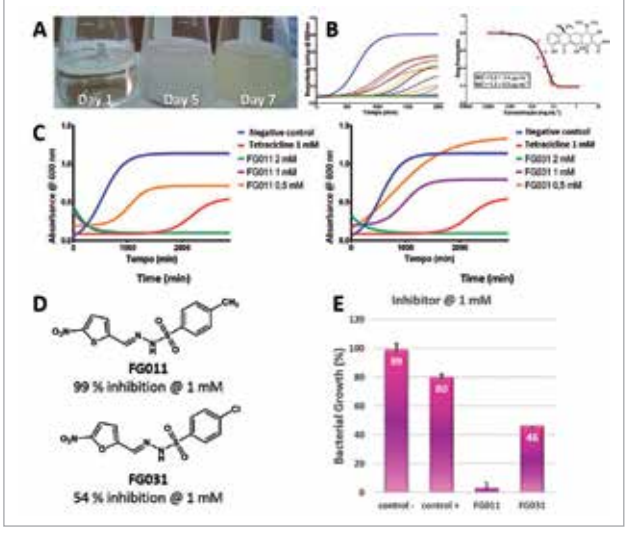

Figure 2. (A) X. albilineans *growth in XAS culture medium. (B) Tetracicline as positive control for* X. albilineans *inhibition. (C)* X. albilineans *phenotypic assay for screening compounds as agrochemical candidates. (D) Representative example of nitro derivatives as* X. albilineans *inhibitors. (E) Inhibitory activity of nitro derivatives against* X. albilineans

Sustainable development exhibits as main feature the use of scientific knowledge to generate technological innovation to meet human needs while preserving the environment. The environmental benefits obtained from the production and use of sugarcane derivatives boost the development of methods and strategies to enhance the generation of bioenergy. However, the occurrence and severity of plant diseases, such as leaf scald, hinder the productivity of sugarcane crops. Sugarcane leaf scald is a widespread and devastating disease caused by the bacteria *Xanthomonas albilineans*. The disease has a dramatic impact on crop productivity, including reduced yields and drop in quality of the juice. Currently, there is no chemical or biological treatment for disease control. Therefore, there is an urgent need for new effective and selective agrochemicals with low cost and environmental impact. This work aims at identifying and developing new molecules with antibacterial activity on the basis of the screening of natural and synthetic compounds. Additionally, a combination of state of the art methods and strategies in structural molecular biology and medicinal chemistry is employed in the design of the bioactive compounds. This work relies on the multidisciplinary organization, which led to the establishment of effective collaboration. In addition, the project provided the opportunity for the creation of a new field research in medicinal chemistry focused on the discovery and development of new agrochemicals for tropical agriculture. The new field of research expands the scope and adds value to the set of projects conducted in Center for Research and Innovation in Biodiversity and Drugs (CIBFar/CEPID) and offers the possibility of our group contribute to the sustainable development of Brazil through effective scientific actions in the strategic areas of agriculture and bioenergy.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Leaf scald is a serious sugarcane disease and the absence of chemical or biological agents against the phytopathology boosts the research of bioactive molecules as effective agrochemicals. *X. albilineans* produces a family of phytotoxins (albicidins), which play an important role in pathogenesis. Mutants of *X. albilineans* lacking the production of albicidins fail to cause any disease symptoms. Hence, enzymes involved in the biosynthesis of albicidins are attractive molecular targets for disease control. Phosphopantetheinyl transferase (PPT) and benzoate-coenzyme A ligase (BCL) play key roles on the biosynthesis of albicidins. Because of that, they were selected as molecular targets for agrochemical design. In addition, we selected several key enzymes of the folate biosynthesis as alternative targets. For instance, N5, N10 - methylenetetrahydrofolate dehydrogenase-cyclohydrolase (FolD) and dihydropteroate synthase (DHPS) act on folate metabolism and are crucial to biosynthesis of thymidine, purines and amino acids. The enzymes were successfully cloned and soluble expressed in large scale. The purification by chromatography led to proteins with high degree of purity (>95%). Enzyme characterization revealed affinities for the respective substrates in the mid-micromolar range. Crystallization assays identified attractive conditions to XaPPT, XaBCL, XaFolD and XaDHPS crystallization. We solved the 3D structure of XaFolD and XaDHPS at high resolution and on the basis of the structural knowledge new inhibitors can be designed (*Figure 1*). Simultaneously, we developed and standardized a phenotypic assay against *X. albilineans* for screening compounds as agrochemical candidates (*Figure 2*). Active compounds from several chemical classes have been identified. In sum, pure protein is crucial to the development of structural biology and structure-based drug design strategies. Additionally, the functional characterization of the targets and the development of a whole-cell method for screening compounds provide the basis for the rational discovery and design of inhibitors as novel agrochemical candidates for leaf scald control.

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ETHYLIC BIODIESEL PRODUCTION USING INTRA AND EXTRACELLULAR LIPASES FROM THERMOPHILIC FUNGI

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FAPESP Process 2008/58077-0 | Term: Oct 2009 to Mar 2012

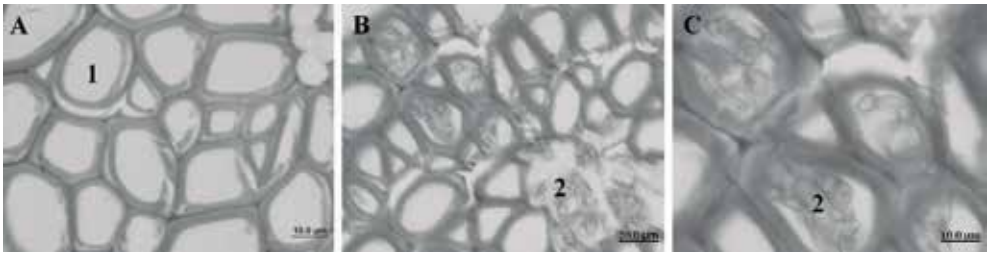

Figure 1. Growth of Thermomucor indicae seudaticae N31 on loofah sponges analyzed by light optical microscope. (A) Sponges without fungi; (B) and (C) whole cells immobilizedin LS; 1. Loofah cell walls appear in dark gray (larger cells) and 2. hyphae of the fungus T. indicae-seudaticae N31 appear in light gray (smaller cells) (Ferrarezi et al. 2014)

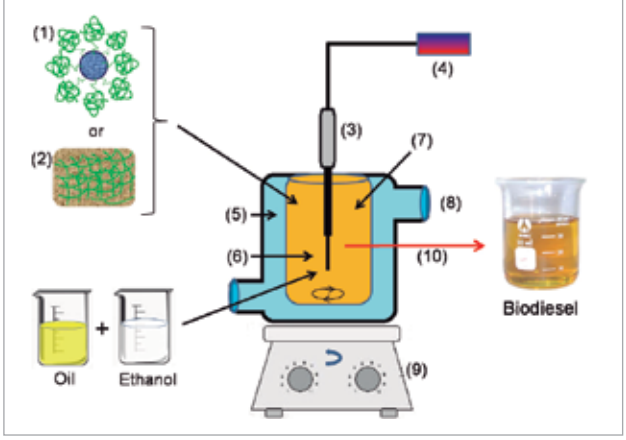

Figure 2. Experimental set-up for enzymatic biodiesel production with ultrasound: (1) immobilized enzyme, (2) immobilizes mycelium, (3) ultrasound probe, (4) power supply, (5) thermostated vessel, (6) reaction vessel, (7) solvent, (8) water inlet/outlet, (9) stirring equipment, (10) Product of reaction after reaction time and removal of glycerol phase (Borges, 2012)

The proposed project aimed to study the use of lipases produced by thermophilic fungi on ethylic biodiesel production. The enzyme lipolytic and transesterification ability were valued in its free form and immobilized in polymeric supports and in immobilized fungal mycelia. Lipases were characterized according to specific reactions and its kinetic characteristics. Besides hydrated ethanol, others such as soybean oil, castor oil and animal fat were used as raw materials to produce biodiesel. The solid state fermentation (SSF) for enzyme production were investigated in constructed bioreactors (bench/pilot). Investigations were also conducted aiming the effect of ultrasound irradiation in the transesterification reaction in the production of biodiesel. The final product were characterized by GC-MS, thermogravimetry, calorimetry, and standards methods for biodiesel.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

This study aimed at selecting fungal strains capable of producing lipolytic enzymes with transesterification property to be used for the production of biodiesel via ethylic transesterification. The ability of transesterification of enzymes was evaluated in both free, immobilized on supports and also, immobilized in its own hyphae. The results obtained so far are presented below. Several strains of fungi with esterifying activity and positive for the synthesis of biodiesel were isolated and some fungi have been identified which include: *Thermomucor indicae-seudaticae*, *Rhizomucor pusillus*, *Myceliophtora sp.*, *Thermomyces lanuginosus*, *Fusarium verticillioide*, *Acremonium sp.*, *Aspergillus sp.* and *Thermomyces sp*. Some lipases have already been characterized for specificity and kinetics. Oils from different botanical sources have being used as feedstock for biodiesel production. Different lipases were immobilized in different supports (Quilles et al. 2015), and some had their hyphae immobilized on their own culture medium as *R. pusillus* and *Thermomucor* indicae-seudaticae (Ferrarezi et al. 2014). The enzyme from *Rhizomucor pusillus* was also immobilized (Ferrarezi et al. 2013), and showed 80 and 70% respectively in yield in the conversion of substrates into esters. The analyzes of the esters were confirmed by gas chromatography. A solid state fermentation (SSF) bench bioreactor was constructed and successfully operated for lipase production by the fungus *Myceliophtora spp* (Casciatori et al. 2013). Investigation was also conducted on the effect of ultrasound irradiation in the transesterification reaction in the production of biodiesel from soybean oil and ethanol using the immobilized enzyme from *Thermomyces lanuginosus* and *Acremonium sp* (Borges 2012; Ohe 2011). The results showed that the temperature of 30 to 35 °C and 5% of enzyme were the optimum conditions for the catalysis. The highest conversion values were observed with soy oil. The irradiation of ultrasound (10%) led to an increased conversion of ethyl esters from 92 to 99%, showing a positive effect on the production of biodiesel by the enzymes.

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STUDIES OF CELLULAR SIGNALING AND INDUCTION MECHANISMS OF CELLULASES FORMATION BY THE FUNGUS TRICHODERMA REESEI (*HYPOCREA JECORINA*)

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FAPESP Process 2010/15683-8 | Term: Jan 2011 to Dec 2014 | Young Investigator

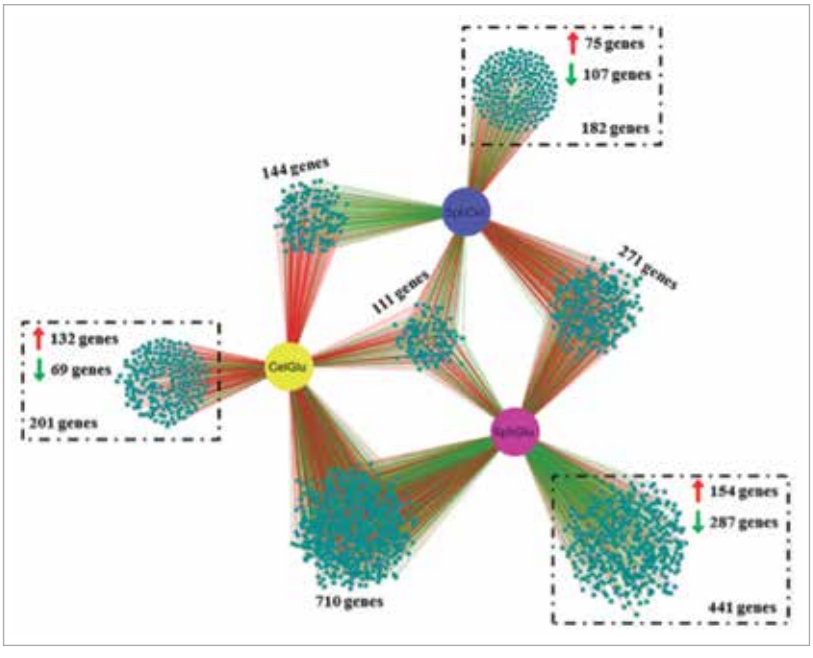

Figure 1. Gene regulatory network (GRN) of 2,060 differentially expressed genes in T. reesei *QM9414 in each tested condition. Cellulose versus glucose (CelGlu), sophorose versus cellulose (SphCel) and sophorose versus glucose (SphGlu). Genes are represented as nodes (shown as squares), and interactions are represented as edges (shown as lines, that is, red indicates upregulated interactions and green indicates downregulated interactions), that connect the nodes: 3,385 interactions*

The filamentous fungus *Trichoderma reesei* (*Hypocrea jecorina*) produces and secretes a large amount of cellulases and hemicellulases that can be used in degradation of biomass components with application in biofuel production. Transcription of the major components of cellulase complex is induced not only by cellulose but also by a variety of disaccharides including lactose, cellobiose, and sophorose and is antagonized by the glucose. However, neither the nature of inducer nor the cellular signaling pathways are totally known. The aim of this project is understand the mechanisms of cellulases formation by the fungus *T. reesei* as well the mechanism of repression and the cellular signaling pathways involved in these process. As approach, genomic and proteomic techniques were used. cDNA libraries were constructed in different conditions cited above and sequenced using the *RNAseq* and expression of the genes differentially expressed were validated by Real Time PCR (RT-qPCR). The secretome and identification of phosphorylated proteins under the conditions cited were analyzed by Differential Gel Electrophoresis 2-D fluorescence (DIGE). With obtained data, a model of global gene expression were constructed using bioinformatics tools what will let a better understanding of gene expression behavior of cellulolytic enzymes produced by *T. reesei* contributing for its application in biofuel industry.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Comparing the parental strain QM9414 transcriptome and mutant strains of *T. reesei* Δ*xyr1* and Δ*cre1* when grown in presence of the inducers cellulose and sophorose as well as in the presence of the repressor and glucose, new components of the regulatory network were identified such as transcription factors, accessory enzymes, transporters, and the participation of XYR1 and CRE1 were also elucidated. Moreover, new potential targets for these transcription factors in the promoter regions were identified and validated by RNAseq experiments. Furthermore, 2D-DIGE analyses identified 30 proteins exclusive to sophorose and 37 exclusive to cellulose. A correlation of 70.17% was obtained between transcription and secreted protein profiles. We also identified by phosphoproteomic analysis 45 possible targets of protein phosphorylation during sugar cane bagasse hydrolysis. Now we are looking for new functions of transporters, mainly sugar transporters and yet not described transcriptional factors.

We hope that these results contribute to a better understanding of the mechanism of induction and catabolic repression in *T. reesei*, increasing the application of this fungus in different biotechnological areas.

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

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FAPESP Process 2008/55914-9 | Term: Jun 2009 to May 2013 | Thematic Project

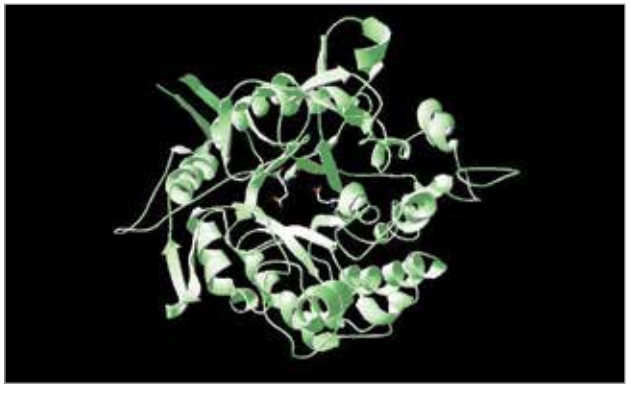

Figure 1. Homology model of Sfβgly. Active site residues are represented as sticks

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

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FAPESP Process 2008/57926-4 | Term: Nov 2009 to Oct 2013 | Thematic Project
co-PI: Maria das Graças de Almeida Felipe

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

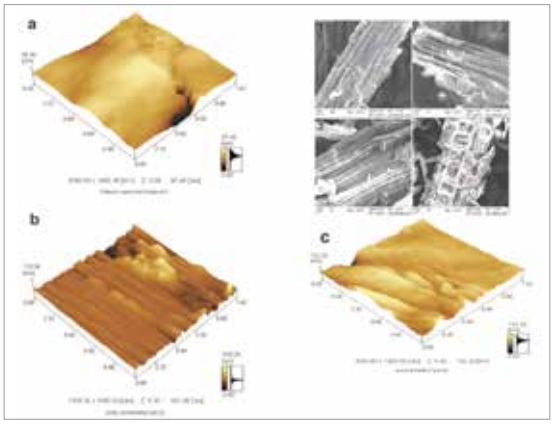

Figure 2. Atomic force microscopy (AFM) amplitude images. (a)Native sugarcane bagasse (b)Oxalic acid-pretreated bagasse (c)Enzyme hydrolysed bagasse and Scanning electron microscopic analysis of sugarcane bagasse. Show surface image of (1) natural, (2) dilute sulfuric acid pretreated, (3) sodium hydroxide pretreated cellulignin, and (4) enzyme hydrolyzed bagasse

The project aimed to accomplish the fractioning of sugarcane bagasse in its main components (cellulose, hemicellulose and lignin) for their use in the production of ethanol.

Acid hydrolysis was used to remove the hemicellulosic fraction followed by alkaline hydrolysis. Enzymatic hydrolysis of the cellulose fraction was then performed. After each stage of fractioning, the solids were characterized by advanced spectroscopic techniques including Raman scattering, infrared absorption with Fourier transformation (FTIR), absorption in the near infrared ray (NIR), thermal lens analysis and photo-acoustic analysis. The use of these techniques was interesting because of their nondestructive character, possibility of *in situ* measurements and potential for further development of compact prototypes. Next, the obtained xylose- and glucose-rich hydrolysates were properly treated through chemical, physical and biological detoxification methods and were used as fermentation media for the production of ethanol by several new xylose-fermenting yeasts and by *S. cerevisiae*, respectively. Xylose-arabinose fermenting species isolated from the Atlantic Rain Forest, Amazon Forest and Brazilian Cerrado ecosystems were tested. Yeasts inhabiting rotting-wood substrates were collected and tested for xylose-arabinose fermentation. This project also aimed to find new species from these Brazilian ecosystems capable of being used in industrial processes.

In all of the unitary operations involved in this project, conditions were optimized by experimental design and data analysis by means of appropriate statistical methodologies. The obtained results allowed the establishment of innovations and advanced technologies that stand to extend the national and international competitiveness of second-generation "bioenergy" technologies for Brazil's national alcohol program. They also allowed the formation of teams and encouraged cooperation among the participant institutions for training and exchange of knowledge.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

This project involved the use of different pretreatments using dilute sulfuric acid, oxalic acid fiber expansion and concentrated aqueous ammonia soaking, which revealed marked hemicellulose degradation and lignin removal from sugarcane bagasse, thus improving the accessibility of cellulases and releasing fermentable sugars from the pretreated substrate. The acid-base pretreatment process was scaled up to 350-L and helped identify potential problems associated with scale-up prior to investing in expensive full-scale equipment.

Structural studies based on spectroscopic principles for characterization of sugarcane bagasse after sequential acid-base pretreatments and enzymatic hydrolysis showed marked changes in hemicellulose and lignin removal at the molecular level.

New yeast species able to utilize pentoses as carbon sources for ethanol production were screened and identified by physiological and molecular methods, and some of these yeasts presented cellulolytic activity.

The cellulosic material showed high saccharification efficiency after enzymatic hydrolysis. Hemicellulosic and cellulosic hydrolysates revealed ethanol production by xylose-fermenting yeasts and *S. cerevisiae* under batch fermentation conditions.

Complementary studies on the influence of agitation, aeration and initial pH were made to optimize ethanol productivity in a stirred-tank bioreactor under controlled conditions. Cell immobilization techniques were presented as a method for improving ethanol production, in this case, a basket-type stirred tank bioreactor and fluidized bed bioreactor that were used to optimize xylose-to-ethanol bioconversion.

The project also demonstrated that electromagnetic field bioreactors present a new and promising research area for improvement of yield and/or productivity in the fermentation process. An electromagnetically fluidized bed bioreactor with cells immobilized in magnetic particles was tested, and the performance of ethanol production of hemicellulosic hydrolysate was affected by the electromagnetic field configuration and intensity. In conclusion, this research generated scientific knowledge regarding methods and perspectives related to the integrated use of sugarcane bagasse for bioethanol obtainment.

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TRANSCRIPTOME ANALYSIS OF ASPERGILLUS FUMIGATUS GROWN ON SUGARCANE BAGASSE

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The biomass of sugarcane is made of lignocellulosic material, primarily hemicellulose and cellulose, polysaccharides which are sugars and high energy that can be converted into ethanol. However, the association between cellulose, hemicellulose and pectin imposes great difficulties in recovering the constituent sugars in the form of monomers with high purity. Due to this recalcitrant characteristic of bagasse, a major challenge in the production of second generation biofuels is the conversion of lignocellulosic substrates in fermentable sugars. In nature, bacteria and fungi play an important role in the degradation of plant biomass, since secrete specific enzymes for the constituent polysaccharides. However, little is known about the response for fungi to different lignocellulosic materials and the production of enzymes and accessory proteins required for the breakdown of plant biomass. The most studied enzymes are from fungi such as *Aspergillus niger* and *Trichoderma reesei*. Other members of the genus *Aspergillus* are able to secrete hydrolytic enzymes of greatest importance, among them the *A. fumigatus*. Although *A. fumigatus* is a pathogenic fungus, is considered an important enzymes producer such as cellulases, xylanases and lipases, whose synergistic effect increases the efficiency of hydrolytic enzymes secreted, but little is known about these enzymes, facts that emphasize the importance of better understanding of this mechanism in *A. fumigatus*. Accordingly, the analysis of the transcriptional profile of *A. fumigatus* when grown in the presence of sugarcane bagasse and the identification of secreted enzymes in the medium (secretome) may bring new knowledge of hydrolytic enzymes that support their industrial applicability, with emphasis on second generation ethanol.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The main objective of this project is the identification and characterization of hydrolytic enzymes from *A. fumigatus* capable of degrading sugarcane bagasse. The *A. fumigatus* conidia were incubated in medium containing fructose (control) and exploded sugarcane bagasse (SEB) and the activity of main hydrolytic enzymes such as xylanase and cellulase were determined by different culture times. We observed an increase of 40X for xylanase (24 h) and 5X cellulase (72h) activities when the fungi were incubated in the presence of SEB. Besides, we observed a gradual increase over the time in reducing sugars as determined by the DNS method, suggesting that hydrolysis in sugarcane bagasse is occurring and monosaccharides are been released in the culture medium. Based on this data, we characterized the secretome profile under the same conditions. The proteins were separated by SDS-PAGE electrophoresis, the bands were digested and the peptides sequenced using a nanoAcquity UPLC system (Waters Corp) coupled to a Synapt G2 HDMS high resolution accurate mass tandem mass spectrometer (Waters Corp.) via a nanoelectrospray ionization source. We identified diverse group of hemicellulases and cellulases including, endo-1,4-beta-xylanase, beta- xylosidase, alpha-1,2-Mannosidase, endo-arabinase, 1,4-beta-D-glucan cellobiohydrolase, alpha-galactosidase and many other ones. Enzymes involved in lignin degradation like laccase, isoamyl alcohol oxidase and etc., were also identified. In addition this study identified several peptidases and proteases, which can be directly or indirectly associated with sugarcane bagasse hydrolysis.

The transcriptional profiles have been characterized through RNAseq techniques. RNAseq libraries were generated from RNA extracted from both cultures and a reference transcriptome was generated by assembly of all Ion Torrent PGM sequencing data. Raw reads (3,000,430 in total) were initially filtered for host nucleotide contamination and ribosomal RNAs. The sequences reads were analyzed using TopHat2 program. The calculation of the difference of expression, normalization and data analysis were performed through the Edger program. From these data, we identified a few genes expressed only in SEB, such as those coding 3 endo-1,4-β-xylanase (AFUA_3G15210, AFUA_3G00470, AFUA_6G13610), endoglucanase (AFUA_7G06150, AFUA_7G06740), cellobiohydrolase (AFUA_3G01910) and carrier hexose (AFUA_6G14560). To get more information about the transcriptome, a new sequencing is being conducted using Illumina platform. For better characterization of the function and activity of the genes and proteins identified, it will be expressed in a heterologous strain, such as of *S. cerevisiae*. Based on these results, it is suggested that *A. fumigatus* has great potential as a hydrolytic enzymes producer that may contribute to the development of more efficient enzyme cocktails and with low cost.

MAIN PUBLICATIONS

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