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ROUTES FOR CELLULOSIC ETHANOL IN BRAZIL

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INTRODUCTION

The climatic changes and the elevation in the costs of the petroleum together with the strategic needs of production of energy have been motivating an unprecedented run towards production of alternative fuels, preferentially from renewable sources. In this scenario, Brazil stands out due to the pioneer use of the ethanol obtained from the sugarcane as fuel since the 1970s.

Besides the tradition, highly selected varieties, sophisticated industrial processes, climate and readiness of agricultural lands guarantee Brazil a comfortable leadership in the production of sustainable ethanol. However, to preserve that position in the currently competitive market, Brazil needs to maintain compatible investments in the generation of new technologies and formation of competences. Nowadays, the conversion of lignocellulose or plant biomass into fermented sugars for ethanol production has been considered as a promising alternative to increase the production of necessary ethanol to meet the world demand.

Cellulose is principal component of the biomass, being the most abundant polymer on Earth. It is formed by a linear chain of glucose molecules covalently linked to each other. Such linkages can be broken to liberate fermentable sugars. However, cellulose is very well protected by the plants, so that they are not easily used by predators. For that reason, the net yield of the conversion of cellulose into free glucose and afterwards into ethanol is not favourable with the current available technologies. The development of technologies to obtain favourable yields will make possible a better use of that rich and renewable raw material found not only in the sugarcane bagasse, but in any other sources of plant biomass (wood, leaves, peels etc.) now wasted or used for less noble purposes. The development of technologies capable to disassemble the plant cell wall requests a deeper understanding of the cell wall structure and physiology from sugarcane as well as of other plant systems. At the same time, the study of enzymatic systems present in microorganisms that feed from cellulose and, therefore, already capable to produce specific enzymes for such a purpose, might help us using the available energy in these polysaccharides.

PERSPECTIVES IN CELLULOSIC ETHANOL PRODUCTION

The ethanol production starting from the sugarcane is accomplished, nowadays, with great efficiency, by fermentation of the sucrose present in sugarcane juice. This process has been named the first-generation ethanol. As opposed to this, the perspective of production of ethanol from cellulose is named the second-generation ethanol.

The research in second-generation ethanol can be divided into four rotes on the basis of the science that will have to be developed in order to produce bioethanol. These routes are 1. chemical hydrolysis – CH; 2. enzymatic hydrolysis – EH; 3. autohydrolysis – AH and 4. pentose-coupled hydrolysis – PH.

In route 1 (CH) the cell walls of sugarcane would be pretreated and subsequently subjected

to acid treatment in order to produce free fermentable sugars. In this case, free-fermentable sugars do not include pentoses. In route 2 (EH) pretreated biomass is subjected (either after acid hydrolysis or directly) to enzyme hydrolysis. Route 3 (AH) is characterized by the use of modified biomass (modified cell walls obtained from a selected variety of genetically modified plants) with enzymes that might or might not have been produced from genetically modified microorganisms.

A negative effect of using acid or basic solvents to loose and break polymers of the plant cell wall to release fermentable mono and oligosaccharides (CH) is the economical and ecological costs of reusing or releasing their residues in the environment. On the other hand, EH phase will demand a larger input of studies and technology to be available commercially. One of the most important bottlenecks in this process will be the production of hydrolytic enzymes/microorganisms selected/modified for that purpose in commercial scale. For what we defined here as the fourth route of the cellulosic ethanol, we expect that microorganisms and plants would be genetically transformed so that very well adapted cocktails of enzymes, produced by transformed fungi, would be used in the process with a raw material (bagasse and/or trash) obtained from genetically transformed sugarcane plants that would have its own cell walls changed to make it more suitable for enzymatic hydrolysis (Figure 1).

Besides the methods of wall hydrolysis, the progress in the knowledge on the physiology of plants used for the ethanol production and the employment of tools of genetic and industrial engineering should play important roles in the increase of the productivity of the ethanol, independently of the phase. However, before detailing some of the aspects of these phases, it is necessary to know what a cell wall is.

The Cell wall

Every plant cell has a wall. It determines the size and the shape of the cell, confers mechanical resistance and protection against the attack of predators and pathogens, delimits the size and chemical-physical properties of the molecules that have access to the interior of the cell, controls the humidity level and it can still work as a storage of minerals, such as calcium as well as carbon and therefore energy. Furthermore, it promotes the adhesion among the cells and is related with shape of the plant organs.

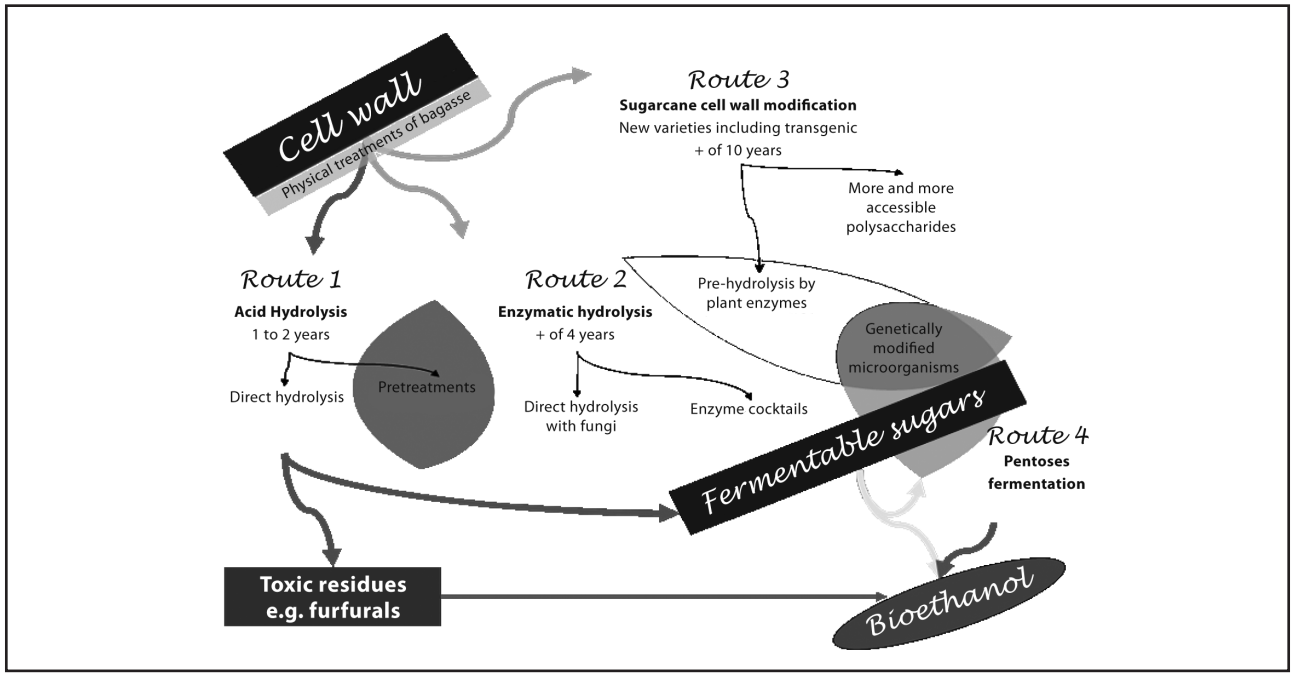

FIGURE 1 Routes to develop cellulosic ethanol.

The cell wall is composed by a polysaccharides mixture, proteins, phenolic compounds and mineral salts. The polysaccharides represent about 90% of the dry weight of the wall and they consist of cellulose, 20% to 40% of the cell wall, hemicelluloses (15% to 25%) and pectins (~30%). That matrix is highly organized and dynamics and could become more rigid or loose rigidity according biological adaptation.

Thirty six cellulose chains are thought to be packed to form a microfibrils, which are long and resistant. The hemicelluloses are a heterogeneous class of polysaccharides classified according to the monosaccharide composition. These molecules are attached to the surface of the cellulose microfibril surface forming the cellulose-hemicellulose domain of the cell wall (Figure 2). The hemicelluloses prevent the molecules of cellulose to collapse, but they also allow a weak interaction among the fibres forming a network. For that reason, they are also called cross-linking glycans (CARPITA, 1993). The cellulose-hemicellulose domain is, in general, submerged in a third domain composed of pectins, a class of highly branched and heterogeneous polysaccharides. Among other functions they are thought to determine the porosity of the wall and, when hydrolysed, their fragments can act as signal for the presence of pathogens and insects (BUCKERIDGE *et al.*, 2008).

The main hemicelluloses found in plants are xyloglucans, glucuronoarabinoxylans and mannans, which are formed by a main chain of glucose, xylose and mannose, respectively. Such main chain can be branched with different monosaccharides (Figure 3). Xyloglucans are the most abundant, being found in most eudicots. Glucuronoarabinoxylans occur in larger proportion in cell walls of grasses (Poaceae) while mannans have a wide distribution, but usually appear in low proportion, except in some groups of ferns (Pteridophytae; SILVA, 2005a). In general, one can say that all the hemicelluloses occur in all plant families, but in different proportions. One important exception is a class of hemicelluloses called mixed linked glucans. They are composed of an unbranched chain of glucosyl residues that are β-1,4 this chain being regularly interrupted with β-1,3 linkages. This hemicellulose occurs mainly in the order Poales (which include Poaceae), how-

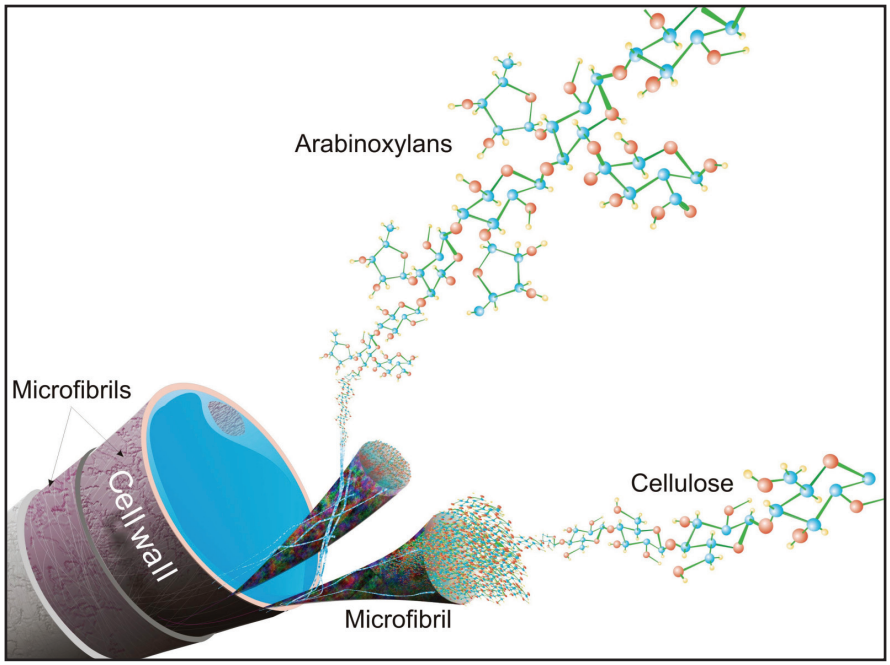

FIGURE 2 Plant cell wall scheme. The figure shows a microfibril structure with 36 cellulose chains. One of the cellulose molecules was prolonged in order to show its fine structure composed by β-1,4 linked glucose residues. One of the hemicelluloses from sugarcane (glucuronoarabinoxylan) also appear in detail with its long xylan chain branched with arabinofuranose and some glucuronic acid unities attached.

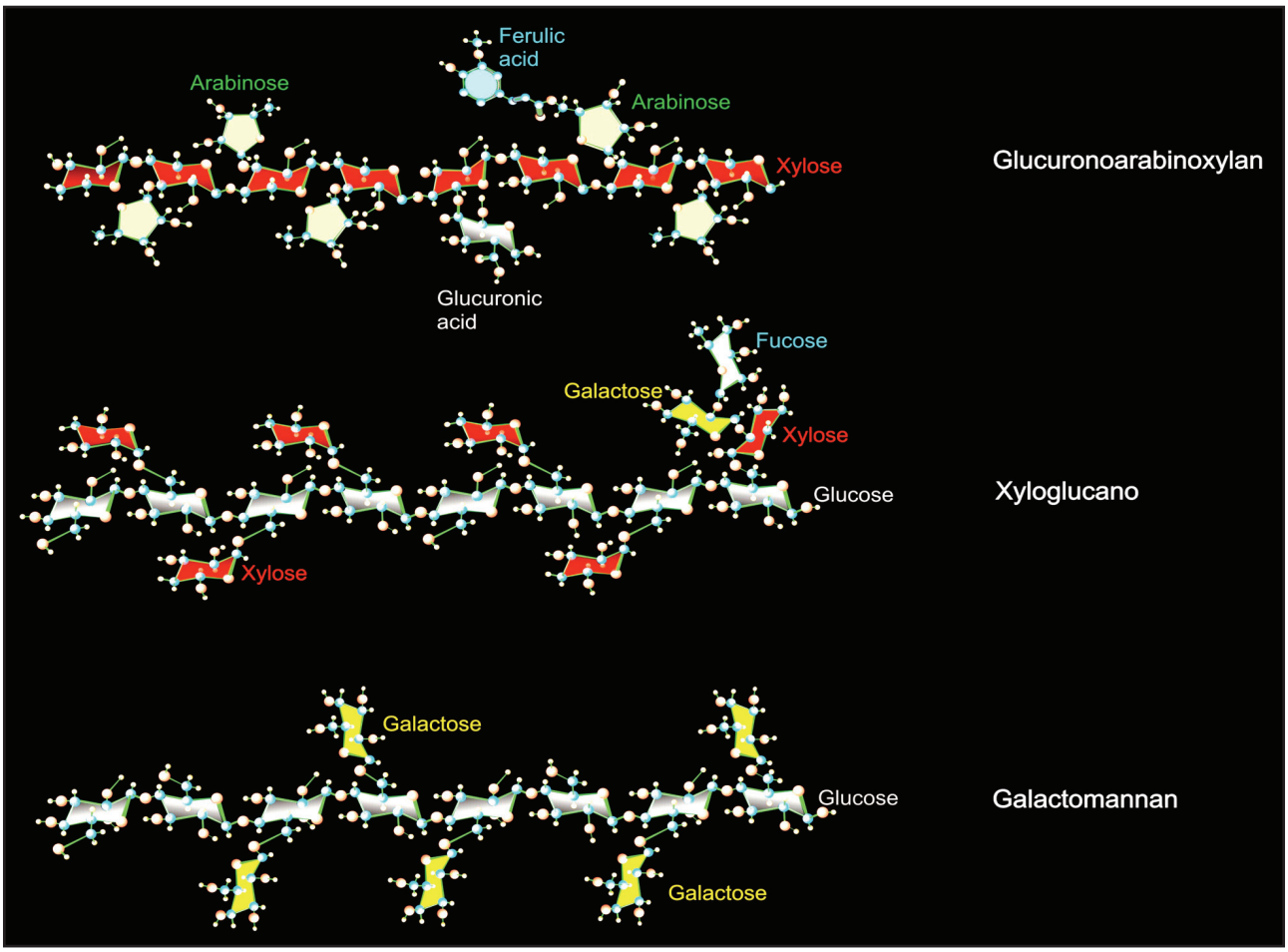

FIGURE 3 Chemical structure of some important hemicelluloses.

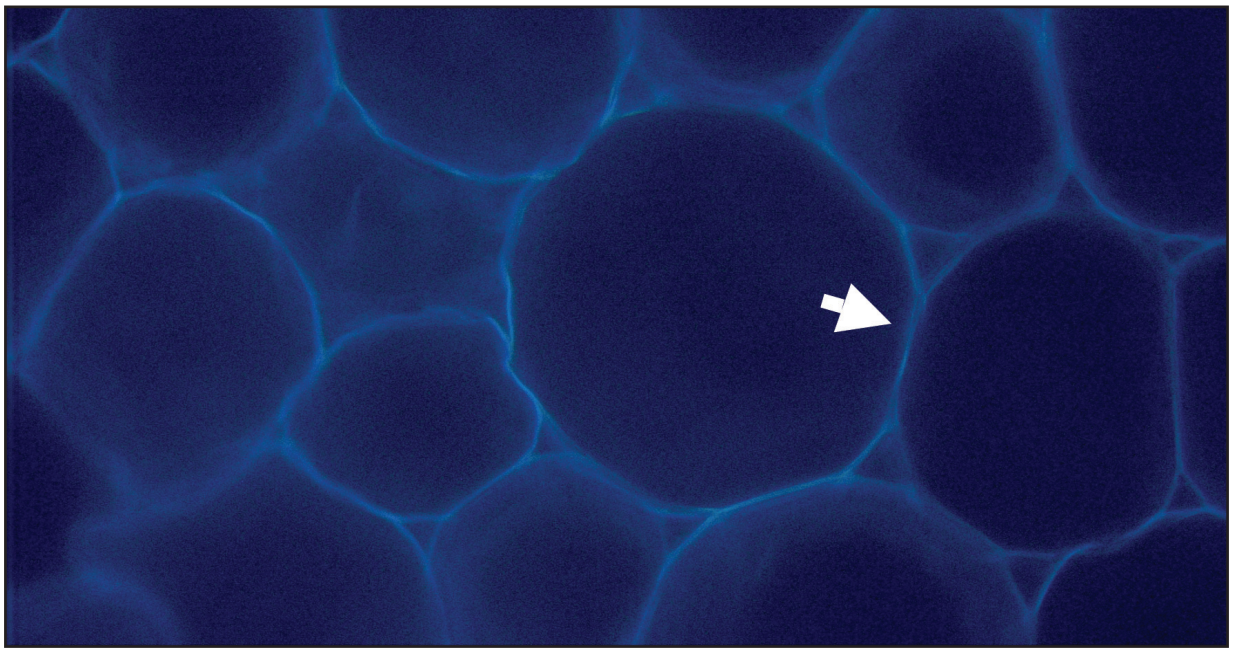

FIGURE 4 Parenchyma cell wall from sugarcane. The white arrow indicate autofluorescence of cell wall due the presence of phenylpropanoids.

ever, they are also present in lichens (an association of fungi and algae) what suggests the genes needed to synthesize mixed linked glucans must be present in most of the species of higher plants (BUCKERIDGE *et al.*, 2004).

Sugarcane cell walls

Sugarcane belongs to the family Poaceae (the grasses), which also includes corn, sorghum, wheat and rice e.g. species of this family display a typical wall architecture that distinguishes them of the other plant groups. While most of the plants have the xyloglucan as main hemicellulose, in grasses is glucuronoarabinoxylans (SAAVEDRA, KAVACSONYI, 1988; SOUZA, 2007) plays this role along with β-(1→3), (1→4)-glucans, although they also have small proportions of xyloglucan and mannan. Glucuronoarabinoxylans and β-glucans are relatively abundant in all the sugarcane tissues, whereas mannans occur in very small proportion (SILVA, 2005b). Xyloglucan appear to be absent, although genes related with its metabolism seem to be conspicuous (LIMA *et al.*, 2001; Crivellari, unpublished).

When examined through fluorescence microscopy, primary cell walls of grasses display auto-fluorescence (Figure 4). This phenomenon is due to phenylpropanoid esterified to some of the arabinosyl residues of the glucuronoarabinoxylans. Ferulic acid esterified to the vicinal polymers may undergo dimmerization, and cross-linking such polysaccharides. Cross-linked polysaccharides are more recalcitrant to enzyme attack (DOS SANTOS *et al.*, 2008, Figure 5).

FIRST GENERATION ETHANOL: THE FERMENTATION OF THE SUCROSE

As mentioned above, the current process of ethanol production from sugarcane is accomplished by the extraction and fermentation of the broth that contains approximately 15% of sucrose (MACEDO, 2008). Before fermentation, performed

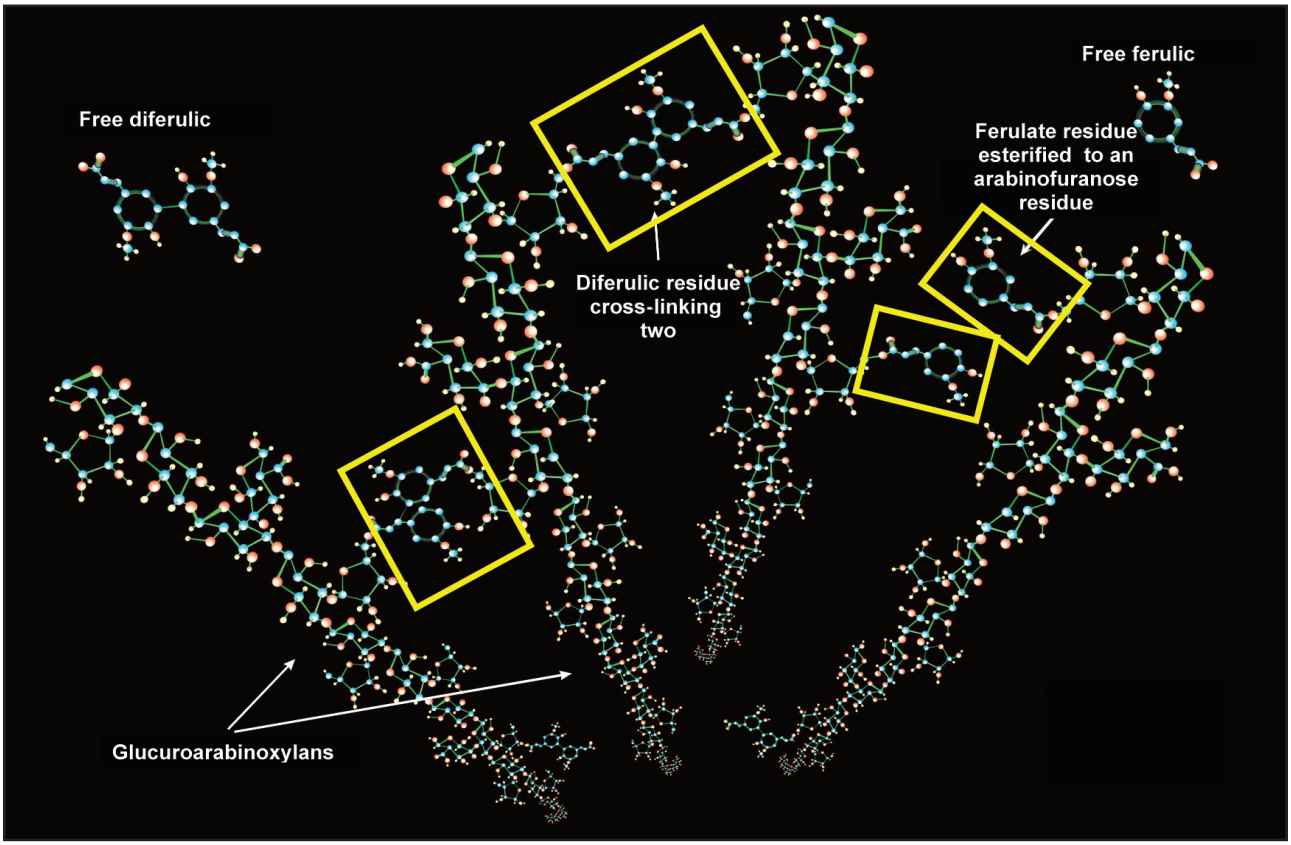

FIGURE 5 Glucuronoarabinoxylans cross-linked by diferulate residues esterified to arabinose of vicinal polysaccharides.

by selected lineages of *Saccharomyces cereviseae* yeasts, the broth is sterilized and purified. The produced alcohol is then separated from the water by distillation. The energy to move the processes comes from burning part of sugarcane bagasse that generates heat and electricity. About 10% of the biomass is burned to produce electricity, whose excess is sold to energy distribution companies. With more efficient techniques of conservation of the energy produced by burning of the bagasse, such excess might reach up to 45%. Furthermore, about 40% to 50% of the trash (straw) of the sugarcane that today is maintained in the field could be recovered and incorporated to the biomass for ethanol production (MACEDO, 2005). This excess of biomass, will be able to be used, in the future, for the production of cellulosic ethanol.

SECOND GENERATION ETHANOL: OBTAINING FERMENTABLE SUGARS FROM CELL WALLS

In order to produce ethanol from plant biomass it is necessary to disassemble the cell wall in order to obtain fermentable monosaccharides. However, it has been previously pointed out how complex the structure of the wall is. Therefore, the process of hydrolysis must to be soft enough to preserve intact the monosaccharide that will be used for fermentation. Currently acid hydrolysis is being studied as a potential form to disassemble the cell wall. Although the process is possible in practical terms, it is not efficient enough to allow the commercial production of ethanol.

The basic process of acid hydrolysis consists in using a strong acid to attack the glycosidic linkages among monosaccharide residues of a polysaccharide. Figure 6 illustrates the process in a simple way. The acids usually applied for the acid hydrolysis in laboratory, are sulphuric, hydrochloric or trifluoroacetic acids. There are advantages and disadvantages in relation to each one. While the sulphuric and hydrochloric acids discriminate little among different glycosidic linkages attacking cellulose and hemicelluloses in a similar way, the trifluoroacetic acid breaks preferentially the weakest linkages such as the alpha linkages present in the hemicelluloses branches.

In grasses the glucuronoarabinoxylan branches are α-linked and these are the first ones to be

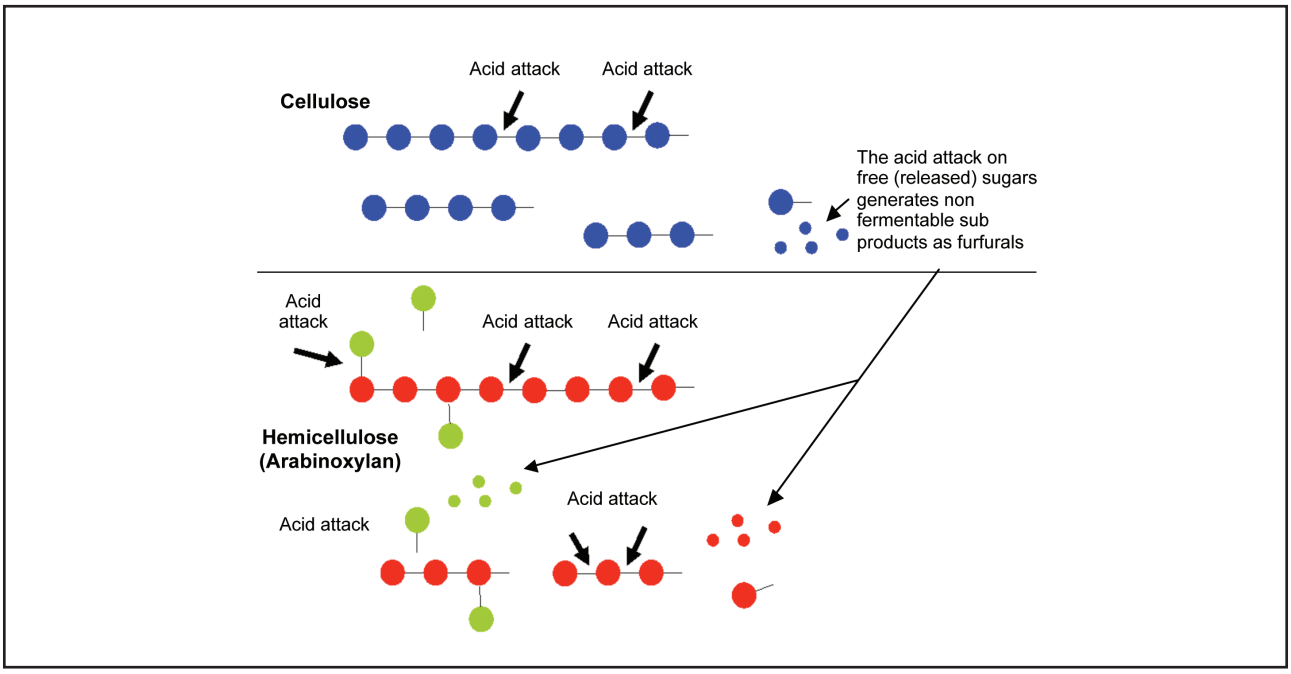

FIGURE 6 Acid hydrolysis of cellulose and branched hemicelluloses as arabinoxylans. Small balls represent products of acid degraded sugars as furfurals and hydroxyfurfurals. Glycosidic linkages are represented by lines between bolls. Each collared ball represents a monosaccharide residue: Blue is glucose, red is xylose and green is arabinose.

broken. Only later, the β-1,4 of xylan main chain are broken. Cellulose linkages are the last to be hydrolysed due the presence of several weak molecular interactions, the absence (or very low level) of water inside the microfibril structure and also because the microfibrils are covered by the hemicelluloses. The problem in a process of polysaccharides hydrolysis containing α and β linkages is that, as the time necessary for hydrolysis is different, the monosaccharides released early in the reaction mixture tend to degrade. This process is sometimes called caramelization (by similarity to the formation of the caramel during the preparation of sugar syrup). If the degradation is very intense furfurals are formed and these compounds are toxic for the yeasts that will be used in the fermentation stage. Thus, by acid hydrolysing a hemicelluloses/cellulose mixture, the temporal displacement among the breaks of the glycosidic linkages of each linkage type becomes limiting for the production of fermentable monosaccharides.

In industrial processes, acid hydrolysis has been accomplished with sulphuric acid (H_2SO_4). However, technical and operational difficulties result in a high cost of the final product. About of US$ 0.80, against US$ 0.35 and US$ 0.27 per kg of ethanol obtained from starch and of the sucrose, respectively. Part of this cost is attributed to the fact that for the hydrolysis to happen in an efficient way it is necessary to heat up the polysaccharide mixture in an acid solution. The temperature is about 100 ºC to 120 ºC and the ideal concentration of sulphuric acid is about of 3% (BUCKERIDGE, 1990). In the specific case of the sugarcane, this cost is minimized by still using a part of the bagasse as fuel.

Another difficulty is related to need of neutralization of the hydrolysed solution in order to carry on the fermentation. In general, for the neutralization, calcium hydroxide (limestone) is used. However, when proceeding in that way, the sulphuric acid is converted into calcium sulphate and cannot be recycled (MARK, 2006). That is the principal factor that contributes to the high cost of the technique. To develop acceptable levels of commercialization (<US$ 0.36/kg) it will be necessary reduce the costs associated with consumption and reuse of the acid as well as improve in the productivity and efficiency in the biomass conversion (KAYLEN *et al.*, 2000; GOLDENBERG, 2007).

ACID HYDROLYSIS

In order to improve the perspective of the use of the acid hydrolysis in commercial scale, the Brazilian company DEDINI – Basic Industry invested in research to make the process most profitable and, now, the company have said to have managed to reach a reasonable level of hydrolysis mixture with the sulphuric acid and ethanol as solvents for lignin, but the costs still above the desirable level. Nonetheless, this kind of research must to allow a reduction in the use of the acid.

Another proposal, made by a group of Chinese scientists, is the substitution of the neutralization process for an electro-dialysis process. It consists in the application of an electric potential among two compartments separated for a semi-permeable membrane electrically charged. Such a process allows an economy up to 55% in the sulphuric acid consumption (CHENG *et al.*, 2008).

Furfurals naturally formed during acid hydrolysis can be used as raw material in production of solvents and resins for fibreglass and other plastic materials. So, its commercialization could become profitable and contribute to reduce the cost of the cellulosic ethanol (RODRIGUES, 2008). Some groups are developing controlled vapour-explosion of the sugarcane bagasse. This technique exposes the fibres, increasing the surface of contact necessary for breaking of the microfibrils as by chemical as by enzyme approaches (PAULO SELEGHIN JR. and GLAUCO CAURIN, personal communication).

Beyond strictly chemical hydrolysis, chemical treatments might to be associated with enzyme hydrolysis. Together, they have a good potential to produce fermentable sugars starting from lignocellulosic biomass. This technology is rather close to becoming commercial and it will be a point of strategic importance for the cellulosic ethanol production. Considering the actual stage of development, and that research continues in the same pace, chemical hydrolysis is expected to reach commercial viability in 2 to 5 years.

ENZYMATIC HYDROLYSIS

In the long term, a commercially viable ethanol from lignocellulosic materials is thought become possible through the use of the biochemical machinery of microorganisms (fungi and bacteria) to disassemble the cell walls. The problem is that, as well as fungi developed strategies to invade the cell wall, plants had also evolved to improve their defence mechanisms. In this way, although there are fungi capable to degrade plant cell walls, the latter are quite recalcitrant to the degradation. One of the ways that grasses developed to resist to the enzymatic attack by microorganisms seems to have been the by the use of ferulic acid – FA cross-linkages among hemicelluloses (Figure 5).

Lignin is an irregular polymer of phenolic alcohols that is quite resistant to the enzyme attack. It accumulates only in secondary cell walls of certain specialized tissues such as fibres and vascular tissues of the plants (xylem). However, in grasses the dimmers formed by FA accomplish a *quasi*-lignification in the whole extension of the cell wall, even in parenchyma cells. Ferulic acid has antioxidant properties which are largely used by the food industry and its presence (together with other hydroxycinnamates) is thought to grant protection against UV light and free radical chain reactions. When reduced to a free radical, it stabilizes the additional electron by resonance, stopping the chain reaction by reacting with another FA (DOS SANTOS *et al*., 2008). In grasses FA is esterified to glucuronoarabinoxylans and once dimerized across the whole cell wall it locks the cell wall distension capability, ceasing cell growth and also resulting in an additional difficulty for microorganisms that may attack the plant. Certain fungi developed feruloyl-esterase enzymes capable to separate the feruloyl residues from the xylans, making the cell wall more susceptible to the attack by xylanases (enzymes able to hydrolyse xylans).

To reach cellulose, that is the principal compound of the cell wall, the fungal still needs to hydrolyse the other hemicelluloses that interact with the microfibrils. For that reason, fungi such as *Trichoderma* and *Penicillium* produce large arsenals with more than a hundred glycosidases and dozens of different cellulases, proteases and lipases, to name but a few. In order to develop an effective technology to convert the wall polysaccharides into fermentable sugars and subsequently to ethanol, it will be strategic to understand the processes related with the attack of each enzyme and enzyme sets about each linkage in the cell wall. However, for that strategy to be really efficient, the fine structures the polysaccharides and the hydrolytic enzymes will have to be carefully studied.

There are some key steps that can help us to guide the route for a third route of cellulosic ethanol production:

a) There are a great number of studies with microorganism enzymes showing how they attack polysaccharides of cell wall. Two roads have been taken in parallel to maximize enzyme production and to understand fungi performance: one is search for more efficient species and other is the genetic transformation of fungi. Beyond improving large scale production of enzymes and heterologous expression of proteins, such knowledge will also help to figure out the industrial hydrolysis process.
b) There is some knowledge on the structure of glycosidases of cell walls of fungi and plant itself. Such piece of information we make us able to design improved enzymes.
c) We know the glycosidic linkages that must to be broken in order to liberate monosaccharide (SILVA, 2005b). Starting from those data and obtaining a complete map of the cross-linking patterns among cell wall polymers we can begin a systematic and detailed scrutiny of enzymes and methods to obtain a maximized hydrolysis of lignocellulosic biomass.
d) We partly know the identity of sugarcane genes related with the metabolism of synthesis and degradation of the cell wall in the sugarcane (LIMA *et al*., 2001). Among these genes there are several capable to degrade the cell wall. Complete identification of genes identity and expression patterns will help us to obtain the control of those genes making

us able to activate them at the desired moment during the process. On the other hand, such kind of information might help to design rational (optimized) hydrolytic processes with adequate enzymes in a logical order rather than simply adding an enzyme cocktail to the lignocellulosic suspension.

Search, selection and engineering of fungi and enzymes

Studies accomplished with sugarcane straw (trash) indicate that *Aspergillus terreus*, *Cellulomonas uda*, *Trichoderma reesei* and *Zymomonas mobilis* might be useful for degradation of the lignocellulosic material (SINGH *et al.*, 2008). Brazil, with its high biodiversity has a large potential to find new and interesting microorganisms. For example, our group have been assaying the cellulolytic activity of more than 50 species of fungi of the soil of the Brazilian savannah (cerrado) and still works in on selected species for industrial use as *Penicillium citrinum* (data not published). Other important strategy is to understand and control the expression of enzymes produced by fungi. Gustavo Goldman (USP-RP/CTBE/INCT by Bioetanol), recently discovered that *Aspergillus niger* species, for instance, has only one transcription factor (XLnR) that regulates the expression of all the genes related to the polysaccharides degradation, while in *Trichoderma*, other transcription factors are important for these same genes (personal communication). Goldman intends to manipulate the mechanisms of regulation of the genetic expression in order to obtain mutants capable of continuously produce enzymes in the presence of substrate, without genetic expression undergoing retro-inhibition by the products of the enzymatic action, as happens naturally.

Another type retro-inhibition or inhibition by the product occurs at the level of the catalysis. The probability of products to adhering into the catalytic site of the enzyme scales with the product concentration. The activity cannot only stop completely, as it might even revert to the reaction direction. In Brazil a group has been working with an approach that consists in stick the glycosidases onto the hemicellulose in order to reduce the local concentration of the enzyme products. To do that, the group of Sandro Marana is trying to link a cellulose binding domain – CBD to hemicellulases (personal communication). CBD are an enzyme domain found in some cellulases that sticks to the microfibril provoking a local change of conformation (i.e. decreasing crystallinity) so that catalysis becomes more efficient. A protein "arm" positions the catalytic site in the exact place where the disorder promoted by CBD exposes the cellulose molecules to the enzyme attack. As the products are soluble whereas the substrates are not, the high local substrate/product ratio confers an additional advantage to the enzyme attachment when compared to soluble enzymes activities.

The amino acids positioned out of the active site of the enzyme define the specificity of the associated enzymatic action. There are two approaches used to study the relation between the structure and function of enzymes in order to afford an accurate approach of engineering of enzymes. A bottom-up strategy is accomplished by a methodology named directed evolution. Small variations in the amino acids sequence produce enzymes with different properties. Variants, whose catalytic efficiency of interest increased, are selected and the subjacent structural variations are analysed. A top-down strategy is the structural study of enzyme families. The different glycosidases are clustered in families. However, the great majority doesn't have their tertiary structure elucidated, or has only few representatives whose structures were elucidated. The groups of Igor Polikarpov (USP-Scar-INCT by Bioetanol) and Munir Scaff (UNICAMP/INCT by Bioetanol) among other groups are studying the tertiary structure of enzymes by crystallography and atomic modelling. They have already managed to crystallize and to solve the structure of xylanases of *Trichoderma reesei* (GALUBEV *et al.*, 2000; ROJAS *et al.*, 2005). They are focusing their work on the creation/enhancement of models of protein folding that can be used to foresee the tertiary structure on the basis of the sequence of amino acids and that can be used in the engineering of enzymatic catalysts.

Using the knowledge on physiology, biochemistry and molecular mechanisms to improve the access to cellulose

Although the basic structure of the hemicelluloses is well known (composition and proportions among the glycosidic linkages), it is very important that we study their fine structure (i.e. the precise positioning of the branching on the main chain of a polysaccharide or the precise distribution of different linkages in the main chain) because the properties of hemicellulases are strongly related with the fine structure.

Specific enzymes might be used in order to reveal the patterns of branching and/or distribution of the glycosidic linkages in the main chain (proportions among linkages β-1,3 and β-1,4 in mixed linked glucans and among the mannoses and glucoses in mannans etc.). We discovered that cellulases of *Trichoderma* can work as a restriction system similar to the restriction enzymes used on DNA, but acting on the fine chemical structure of the hemicellulose polysaccharides (TINÉ *et al*., 2003, 2006). Beyond helping to elucidate the fine structure of polysaccharides, this kind of Information is valuable in order to understand catalysis mechanisms and, thus, to set up a research platform dedicated to increase the efficiency of those hydrolases.

Our group has also been studying the composition and fine structure of cell wall in organs of sugarcane during development. We have been investigating the role of phenolic compounds in the recalcitrance of the cell wall to hydrolytic attacks by inhibiting enzymes from phenylpropanoid pathway in different intensities with metabolic modulators. By evaluating the susceptibility to xylanase and cellulase, we found that very low reduction in cell wall phenylpropanoid content make the bagasse more accessible to enzyme hydrolysis (data not published). We are now developing a new methodology in order to modify lignocellulosic material in large scale without the need genetically engineer plants and subsequently licence them as a new OGM. We named this approach *physiological engineering*. This has the advantage to putatively permit the improvement of bagasse properties for a better hydrolysis in any cultivar of sugarcane.

The global climatic changes are the great propeller of the search for renewable fuels and our group studies the effects of environmental changes on the sugarcane and species of interest for renewable energy in Amazon areas, as *Senna reticulata* and *Euterpe oleracea*. Our objectives are to understand the mechanisms of biomass accumulation and to gauge the potential to responses of these plants to climatic changes.

Sugarcane plants incubated in atmosphere of CO_2 of 720 ppm (expected concentration for 2050) presented an increase in the photosynthetic rate and an increase in biomass about 60% in the culm (DE SOUZA *et al*., 2008). In these plants, we observed the super-expression of genes related with cellular expansion as xyloglucan endo-transglycosilase – XTH, with the photosynthesis and with the inhibition of the expression of genes related to the phenylpropanoid pathway (intermediary of the lignin biosynthesis). Parallel, the group of GLÁUCIA SOUZA, with which we collaborate, observed that plant with high productivity of sugars express XTH with larger intensity and they have also been inhibited expression of the cinnamil orto-methyltransferase – COMT, from the phenylpropanoids synthesis.

At this time we are performing the studies on the role of XTH in the cell wall metabolism and the process photosynthetic in sugarcane. An increase the productivity of the sugarcane by inducing the expression of the photosynthesis genes that answer to high CO_2 may be expected. With this, the plant could not only produce more sucrose, but also more cellulose even in low CO_2 (see more details in the Chapter 6).

In order to manipulate the plant enzyme machinery to self-disassemble the cell wall, we also need deepening our knowledge on signalling mechanisms and expression of genes related to the cell wall in sugarcane. A recent work from our lab (unpublished) showed that a gibberellin plant hormone interferes with wall expansion and sugar metabolism in seedlings of sugarcane. This seems to shed some light into ways to manipulate plant metabolism towards modified walls.

SUGARCANE DESIGNED FOR AUTOHYDROLYSIS

Our group is focused in understanding both the relationship between the expression of the genes related to the biosynthesis and hydrolysis of the cell wall as those related with the carbohydrates metabolism in general. In 2001, we participated of the SUCEST (sugarcane expression sequence tags) project and found 459 genes related to the metabolism of cell wall (LIMA *et al*., 2001). Whereas genes related with cell wall degradation are hardly expressed during the growth, great part of the genes involved with cell wall biosynthesis appear to be fully activated, what suggests that the plant is growing and cells are expanding. However, these hydrolase genes must be expressed in specific conditions as the leaf senescence, building of plasmodesmata, and other kind of differentiation. If we obtain control of the expression of these genes we could induce the plant to express them and produce the enzymes at the time as the crop or in inactive forms that might be triggered in the planta. This will reduce the need to introduce fungi enzymes. We also would induce sugarcane plants to express heterologous genes that would become active under certain conditions, during the processing of biomass. In another study, an endoglucanase E1 of *Acidothermus cellulolyticus* that optimally works in 81 ºC and pH 5 was expressed in the apoplast of several plants including *Arabidopsis* and rice. The enzyme was activated in raw extracts after the crop and after an acid pretreatment, but it didn't cause degradation of cellulose *in planta* (DAI *et al*., 2000). However, studies in our laboratory indicate that the presence of hemicelulose and phenylpropanoids might prevent the access of E1 to cellulose. Hence, it is important to study what enzymes are necessary to break lignin (lacases and peroxidises), as well as hemicelluloses (feruloyl-esterases, xylanases, lichenases etc.). Later on, the heterologous expression of those enzymes can be studied previously in *Arabidopsis* or *Physcomitrella* or directly in sugarcane, addressing them for cellular compartments in order to obtain their action in the suitable moment. As sugarcane presents polyploidy its transformation and the long term stability of the heterologous gene is not easy to obtain. However, Helaine Carrer and from (ESALQ/INCT by Bioetanol), and at least one enterprise Allelyx, has been working in sugarcane transformation for a long time and has already obtained good results.

Simultaneously with transformation development, we need to search for hydrolytic enzymes of cell wall produced by sugarcane and other plants of interest as well as the signalling mechanisms involved in regulation of the expression of those genes in order to control those signalling mechanisms. Our group, in collaboration with Edivaldo Ximenes (University of Brasilia (UNB)/INCT by Bioetanol) and Maria de Lourdes Polizeli from USP-RB/INCT by Bioetanol, is investigating ideal conditions (which enzymes are produced, how long it takes to produce, in which order and proportions, pH and temperature optima, and how to avoid inhibition by the reaction products etc.) to digest sugarcane bagasse with the highest efficiency.

Another approach that can be called energy-cane project is to induce modification in types and amounts of hemiceluloses present in the wall controlling the amount of lignin in order to maximize production of polysaccharides convertible in ethanol. Such a plant could be used for hydrolysis with enzymatic cocktails of high efficiency, genetically modified fungi or even for enzymes expressed by the plant itself. In spite of the high degree of knowledge we need to obtain in order reach this goal, the cell wall manipulation and introduction of genes is, in fact, possible. We believe that these fourth routes of ethanol technology production will become viable in about 10 years. Together with these "pro-ethanol modifications", we must deep our knowledge and develop improvements in sugarcane physiology in order to prepare cultivars able to grow in non-ideal environments both to attend expansion necessities as well as to adapt it to changes waited to occur because of global warming.

In order to reach these goals, we need to prioritize research lines as the complete sequencing of sugarcane genome and some key-fungi such as (*Trichoderma*, *Aspergillus* and *Penicillium*), mapping of the wall-related genes, mechanisms of physiologic control (hormones, transcription factors), as well as in the structure and efficiency of enzymes.

Figure 7 summarizes the four phases of ethanol technology development integrated to each other.

FERMENTATION OF PENTOSES

Sugarcane hemicelluloses are rich in xylose and arabinose. *Saccharomyces cereviseae*, the microorganism employed in the production of alcohol starting from sucrose, has very low efficiency in fermenting pentoses. The presence of pentoses in fact inhibits the fermentation of the hexoses by *S. cereviseae*. A perspective is the prospection and use of other species of fungi, better adapted to fermentation of pentoses. Species such as *Pachysolen tannophilus* are capable to use xylose efficiently and other pentoses (less efficiently) after they consuming the glucose and cellobiose available (HINMAN *et al.*, 1989).

USING OTHER SOURCES OF BIOMASS FOR CELLULOSIC ETHANOL

The reason why sugarcane bagasse should be a priority in research programs to study cellulosic ethanol in Brazil is the existence of several mills that are adapted to use sugarcane as raw material. But in a future scenario, in which the technological barriers to obtain large scale production of ethanol starting from lignocellulosic ethanol will be overcome, other sources of raw material might come into the scene.

The eucalyptus is now commercially the largest source of cellulose available, although its production is directed to papermaking. However, the bark of eucalyptus, now wasted, may be an interesting carbohydrate source that can be used in the future as raw material for production of cellulosic ethanol. Some years ago, our group surveyed the composition of the cell wall of the eucalyptus in a project financed by a paper company Suzano Papel and Celulose LTD. Our group has already fractionated eucalyptus bark and found that they have a typical type I cell wall, with higher amounts of pectins and xyloglucan as the main hemicellulose. The group led by Carlos Labate has also performed studies of carbohydrates in the bark from different eucalyptus varieties. They found that some barks are hexoses rich presenting up to 5% of sucrose in its composition. Labate is also

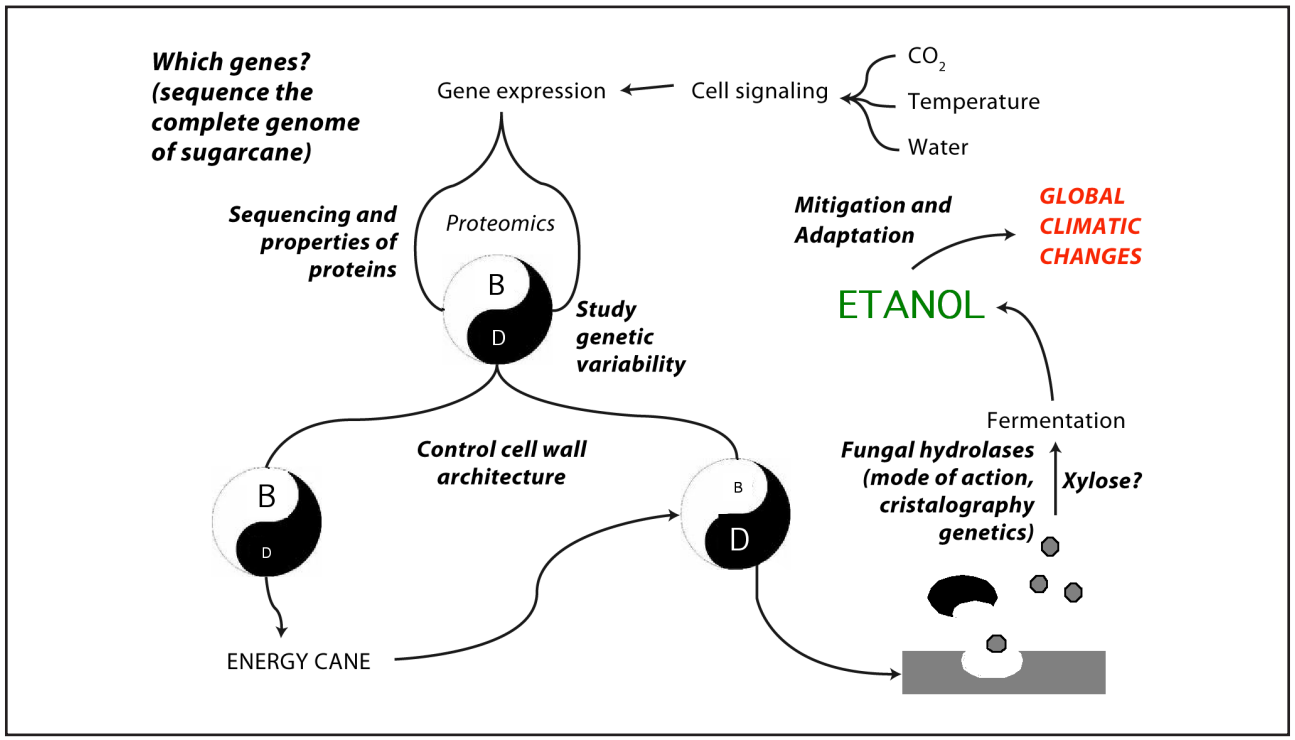

FIGURE 7 Routes to cellulosic ethanol using sugarcane bagasse.

interested in studying cellulases with specificity for those raw materials, as those found them within the digestive treatment of termites and other wood specialized insects (personal communication). Besides the knowledge on the composition of the eucalyptus cell wall, the pulp was already studied with relationship to the attack of fungi enzymes (MEDEIROS *et al*., 2002).

Coffee dreg is another rich source of polysaccharides. It is rich in mannan and cellulose that might be digested by a mannanases and cellulases (LISBON *et al*., 2006). There are several other possibilities, but mentioned above can be seen as examples that could complement and eventually overcome the production of sugarcane-based ethanol. The peduncle of the banana bunch is a residue that can be obtained in relatively great amounts and has composition similar to sugarcane bagasse (not published) and can be digested by a similar mixture of fungi enzymes (MEDEIROS *et al*., 2000).

Even with the focus on the ethanol of high technology, it is important that we do not forget to give some attention on the physiology of other plants of interest for ethanol production, be it from sucrose, starch or cell walls. We needed to invest in plants to increase productivity and precocity in order to reduce the need of expansion of the planting areas. In the same way, it is important produce varieties that are more resistant to the drought, elevated temperature, cold and pathogens in order to adapt to the effects of the global climatic changes in course, as well as to assist to the demand for varieties capable to be cultivated in new areas with non-ideal climate as the traditional ones.

Our group are also investigating the mechanisms of natural cell wall digestion in both fruits and carbohydrate storage seeds (by far the most efficient systems know to degrade cell walls and use the sugars as sources of energy) by determining enzymes involved, the expression sequence and the composition and structure of the cell wall of seeds in order get hints that help us to design an optimized process of hydrolysing cell wall and seeking to the production of alcohol through the maintainable use of native seeds.

Seeds of species native to several Brazilian biomes, among them the Atlantic forest and of the Savannah, accumulate great amounts of cell wall polysaccharides (BUCKERIDGE, 1990; BUCKERIDGE *et al*., 1995; MAYWORM *et al*., 2000). In some cases, it is possible to extract carbohydrates in large quantities, possibly for industrial scale. We have, for instance, developed a process to obtain galactomanan starting from *Dimorphandra mollis* seeds (PANEGASSI *et al*., 2000). Thus, a possible strategy for bioenergy production would be to develop technologies to co-produce an eco-ethanol starting from reserve polysaccharides of some native species seeds from savannah and Atlantic Forest. Our group has been studying these biological systems for many years (see BUCKERIDGE *et al*., 2000 for a review) and we believe that the development of technologies to produce bioethanol from storage cell wall polysaccharide from seeds of native tree species would stimulate the use of agroecosystems in association with sugarcane crops and restored native biomes. At the same time, we would like help to protect these biomes from destruction due crop expansion. Through the last 15 years we have been studying the biochemical and physiological mechanisms involved in the processes of degradation of those polymers. We purified several enzymes (BUCKERIDGE *et al*., 2000) and cloned related genes (ALCÂNTARA *et al*., 1999, 2006; LISBON *et al*., 2006; BRANDÃO, 2009), as well as investigated the hormone control mechanisms of cell wall degradation in those study models (SANTOS *et al*., 2004; TONINI *et al*., 2006).

The idea is to introduce genes in fungi, bacteria or yeasts to make them able to express hydrolases that attack galactomannans and xyloglucans from legume seeds. Then, we could use the yeast to degrade the polysaccharide of the seeds to produce monosaccharides and, to proceed, to promote the alcoholic fermentation. Indeed, some of the most interesting species to be used for this purpose would be *Dimorphandra mollis* (40% yield) and *Sesbania virgata* (21% yield) of galactomannan and *Hymenaea courbaril* (40% yield) of xyloglucan (BUCKERIDGE *et al*., 2000).

The amount of ethanol to be produced it is relatively small in relation to sugarcane production and potential. However, the environmental

advantages of regenerate forests allied to the sugarcane plantations are huge. This strategy, we denominated The Midway (BUCKERIDGE, 2007), has the potential of producing an ethanol that could be certified as low environmental impact process and assure some market slices.

FINAL CONSIDERATIONS

The cellulosic ethanol, beside the biodiesel, is a promising source of sustainable and efficient fuel able to support a significant part of the universal demand for liquid fuels, such as the propelling of vehicles as well as to feed fuel cells. Several methods for obtaining ethanol are in experimentation and they are all important in order to Brazil to keep its leadership in this field so that the technological value of the biofuels can be reverted in our favour while the product moves forward to become a commodity. In this route, the sugarcane blunts with large advantage being the plant on which we must deposit our largest efforts in short term. By developing completely a viable technology to produce ethanol from cellulose, we should be able to adapt the technology to convert virtually any source of biomass in free fermentable sugars to produce ethanol and other unforeseeable applications. At the same time as the focus should be placed in the development of technologies related to pretreatment, hydrolysis, fermentation and distillation, we need also to keep research on the improvement of crops, including their physiology and agronomy.

Bioenergy production is an attitude of extreme importance for us to face the serious environmental challenges related with the effects of the global warming. Although the bioenergy is not the only solution for this problem, it certainly will contribute to mitigate the emissions of fossil fuels.

Another equally important challenge is the preservation of biodiversity. The productivity increase expected in the next 10 to 15 years, should be used to reduce the need of crop expansion to produce fuel. At the same time, the recovery of forests should be encouraged, and, if possible, they can occupy space amid the sugarcane plantations and help to produce energy.

The production of ethanol from cellulose with high efficiency and sustainability will not be task of a few scientists, but the result of the integration among several research groups specialized in different areas of the physiology, ecology, biochemistry, genetics, enzymology, physics, chemistry and engineering, to name but a few. The industrialized world is gradually changing its energy matrix. It is a event perhaps without similar in the history and Brazil is one of the leaders. We can develop a new technology, reduce the production of greenhouse gases and, at the same time, use it to recover the biodiversity, integrating sustainability and technological development. Perhaps it is not too much to say that Brazil has the chance of leading a transition among old *Homo sapiens sapiens*, a powerful but pollutant species, towards to a *Homo sapiens ambiens*, a new and still more powerful and balanced species.

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