Bioresource Technology 101 (2010) 4820–4825

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Bioethanol from lignocelluloses: Status and perspectives in Brazil

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ARTICLE INFO

Article history:
Received 1 September 2009
Received in revised form 13 November 2009
Accepted 16 November 2009

Keywords:
Second generation bioethanol
Brazilian bioethanol program
Sugarcane
Bagasse
Hydrolysis

ABSTRACT

The National Alcohol Program – PróAlcool, created by the government of Brazil in 1975 resulted less dependency on fossil fuels. The addition of 25% ethanol to gasoline reduced the import of 550 million barrels oil and also reduced the emission CO_2 by 110 million tons. Today, 44% of the Brazilian energy matrix is renewable and 13.5% is derived from sugarcane. Brazil has a land area of 851 million hectares, of which 54% are preserved, including the Amazon forest (350 million hectares). From the land available for agriculture (340 million hectares), only 0.9% is occupied by sugarcane as energy crop, showing a great expansion potential. Studies have shown that in the coming years, ethanol yield per hectare of sugarcane, which presently is 6000 L/ha, could reach 10,000 L/ha, if 50% of the produced bagasse would be converted to ethanol. This article describes the efforts of different Brazilian institutions and research groups on second generation bioethanol production, especially from sugarcane bagasse.

1. Introduction

The indiscriminate use of fossil fuels by the mankind, especially since the dawn of modern civilization has led the world to a unique situation at present. As a consequence of this, the emissions of CO_2 in the atmosphere are being viewed responsible for causing extensive climate changes (Buckeridge et al., 2009). In the 1970s, Brazil started a program to substitute gasoline by ethanol in order to decrease the dependence from politically and economically variable periods. In this program, sugarcane was chosen as the feedstock to produce ethanol, and as a consequence, agricultural and technological studies were greatly intensified, leading Brazil to a very favorable position in terms of energy security. However, it must be noted that an only part of the biomass produced is used for bioenergy production; one-third of the plant is used for sugar production, one-third is bagasse, which is burnt for electricity production and the remaining one-third is left in the field, which is decomposed by the microorganisms (Cortez et al., 2008). Therefore, a significant increase in ethanol production would be possible only if technologies are developed to convert the polysaccharides from the leaves, straws and bagasse of sugarcane, which represent two-third of the biomass.

Presently, Brazil has more than 80% of its vehicles running with bioethanol and even small airplane engines are now being developed. With the increasing instability in petroleum prices and its supply from the Middle East, many countries have decided to direct their energy policy towards the use of biofuels. This imposes an enormous pressure on the production of crops that can supply bioethanol. However, the use of food crops such as maize, sugar beet, etc. for bioethanol production could cause conflict with food production. The major advantage of the production of bioethanol from sugarcane bagasse in Brazil is the use of a residue, almost 10% of the total generation, which is usually rejected and cause environmental problems.

The Brazilian sugarcane system of agroenergy is considered as the most efficient system (Goldemberg, 2007). Therefore, in order to meet wider needs, a significant increase in the production of

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doi:10.1016/j.biortech.2009.11.067

ethanol would be possible only if the basic knowledge necessary for the development of technologies that will be capable to obtain energy from lignocellulosic materials present in sugarcane is developed. Although the chemical hydrolysis of biomass from sugarcane is a consolidated methodology under laboratory conditions, its large-scale application is not yet economically viable in Brazil. The Brazilian National Institute of Science and Technology in Bioethanol (NIST–Bioethanol) is working to understand the lignocellulosic structure of sugarcane and sugarcane leaves. The aim is to unveil the patterns of gene expression that could be useful to find ways to induce the plants to degrade their own wall and be suitable for subsequent hydrolysis. It is proposed to screen the existing varieties to find the gene markers for identification of plant materials with increased susceptibility to hydrolysis. Also, the tests of mechanical properties of sugarcane for further acid and/or enzymatic hydrolysis shall be performed. At the same time, the search for microorganisms capable of efficiently hydrolyzing the walls and the investigation of enzymes and genes from both microorganisms and sugarcane will be worked on. Overall, the NIST–Bioethanol program is focused in five areas, which are represented by five Research Centers: (1) Center of Sugarcane Breeding; (2) Center of Plant Physiology and Cell Biology; (3) Center of Gene Expression and Transformation; (4) Center of Fungi Prospection for Glycohydrolase Production; and (5) Center of Enzyme Characterization and Process Engineering. These centers are focused in developing biotechnology in order to obtain fermentable carbohydrates from the sugarcane cell wall. With the data from the Centers in hands, it will be possible to provide the fundamental biotechnology knowledge necessary for scaling up studies and further increase of an efficient bioethanol production in Brazil.

2. History of bioethanol production in Brazil

Since the early part of the last century, Brazil started to use ethanol as fuel (Rosillo-Calle and Cortez, 1998; Soccol et al., 2005). The oil crisis and the low prices of the sugar in the 1970s pushed Brazil to the beginning of a new strategy to by-pass this situation. The Brazilian government intended to prevent a slow down in energy consumption in order to maintain the economic growth by substituting imported petroleum by domestic sources as fast as possible. In this way, the Brazilian government started its policy to substitute the gasoline with sugarcane alcohol. The program saw the participation of several groups of people involving politicians, military groups, alcohol industry, sugarcane producers, researchers and the media. The government intensified the use of a mixture of ethanol and gasoline (gasohol) to fuel common cars.

This favorable situation of the alcohol sector indicated the effort made by the government to start the National Program of Alcohol (PróAlcool). The PróAlcool was set up in 1975 when energy supply became a main priority (Rosillo-Calle and Cortez, 1998). When the alcohol program was started, the government, through incentives, created many actions making ethanol a more economically, socially and environmentally acceptable alternate fuel (Puppim de Oliveira, 2002). PróAlcool was described as the answer to the first oil crisis as well as a solution to the problem of the fluctuation of sugar prices in the international market (Moreira and Goldemberg, 1999). Besides, the low prices of sugar, the huge surpluses in sugarcane production and processing capacity led the sugar industries and sugarcane farmers to pressure the government to try to find alternatives to these problems. The use of alcohol appeared to be a good promise.

Brazil is one of the most important agricultural countries in the world. Among its various agro-produces such as coffee, soybean, cassava, corn, fruits, sugarcane, etc., the latter has been one of the main products since last several decades. In 1970, 50 million tons of sugarcane was produced, yielding approximately 5 million tons of sugar. Sugarcane was chosen as the substrate for ethanol production due to a number of reasons, including its great adaptation to the Brazilian soil and weather conditions. In this phase, the anhydrous alcohol was mixed to gasoline using up to 20%. However, to meet the demand, the alcohol production required to increase from 600,000 L/year to 3 billion L/year. However, there was no rigid commitment to supply the alcohol intensively, which led the production to stay at low level. If the sugar prices fell, alcohol could be produced instead and vice versa, leading to variation in production.

In phase two, from 1980 to 1983, the Brazilian government kept subsidizing the vast expansion of sugarcane capacity and industrial investment in mills and distilleries. Automobile factories started to produce only-alcohol-fuelled cars whose technology was developed indigenously (Puppim de Oliveira, 2002; Soccol et al., 2005). This phase was marked by the autonomous alcohol-only processing plants that were built using government subsidies to achieve the agricultural output necessary to fulfill future demand for alcohol. These plants could not produce sugar as an alternative end-product. In both the phases, government interventions were very important to increase alcohol production and consumption. The rapid growth in the production of hydrated ethanol resulted in large surpluses of gasoline which had to be exported, forcing the Brazilian Company Petrobras to make costly changes to the oil refining structure.

Later, in 1984, around 94% of passenger cars were fuelled by ethanol. PróAlcool's credibility began to fall due to a combination of factors such as rapid increase in ethanol-fuelled passenger's cars, stagnation of ethanol production, which was caused by the low prices of ethanol paid to sugarcane growers, political uncertainty toward the programme, the negative attitude of Petrobras, lower costs of imported ethanol and methanol, increased domestic oil production, international political changes after the collapse of the U.S.S.R, and other factors (Rosillo-Calle and Cortez, 1998).

In 1989 the Brazilian alcohol production reached 11.7 billions liters, in the 372 distilleries all over the country. More than 4.5 million cars were fuelled by alcohol only and the other 9.5 billion by a mixture of alcohol-gasoline (Puppim de Oliveira, 2002).

According to a recent report of the Ministry of Mines and Energy, Brazilian ethanol production was 25 billion liters in 2008, of which 5.16 billion liters were exported. This was 45.7% higher than the 3.5 billion liters exported in 2007 and was equivalent to more than twice the exports of gasoline made by Petrobras in 2007 (IBGE, 2009). According to the report, the largest buyer of Brazilian ethanol was the United States, which imported 2.8 billion liters. The Ministry also informed that the increase in biodiesel amount added to diesel (from 2% to 3%) led to a saving of US$ 976 million that corresponded to a reduction in diesel imports of 1.1 billion liters in 2008. The Brazilian car industry became revitalized only with the introduction of flex fuel vehicles (FFVs) in March 2003. FFVs can use various mixtures of alcohol and gas, thus allowing the consumers to react to the different prices signals of the two markets (Hira and Oliveira, 2009).

Currently, there are 448 bioethanol production units installed in the country (Udop, 2009), of which 354 units are located in the south, southeast and center-west. In the Amazon region, which occupies nearly 50% of Brazilian territory, there are only five units. New units of bioethanol production are expected to be installed in future. However, an expansion of the ethanol production from around 27 billion liters in 2009 to 104 billion liters in 2025 will necessitate the reduction of production costs to sustain the transportation from more distant areas within Brazil to internal and external markets. In addition, as one adds more advanced technology to gain greater productivity per unit of land and provide better environmental performance, the complexity of the production process necessarily increases. This almost always brings with it additional costs. Today, a hectare of sugarcane can produce about 6000 L of ethanol with production costs ranging from US$0.25 to 0.30/L (Cerqueira Leite et al., 2009). According to IBGE (2008), around 70% of the ethanol production costs are raw material. In recent years, the programs developed by the Sugarcane Technology Center (Centro de Tecnologia Canavieira/CTC), Ridesa group, and the Cane Center (Centro de Cana) from the Agronomic Institute of Campinas (IAC) have resulted in a sustained increase in sugarcane yield in the order of 1.6% per year. These improvements are expected to continue. However, sugarcane varieties will need to be developed to adapt to new areas of the country, since nearly all the commercial varieties in use in Brazil have been developed for the state of São Paulo, responsible for 62% of all sugarcane produced in the country (Brazilian Ministry of Agriculture and Agroenergy, 2008).

Macedo (2005) reported that about 92% of the bagasse was used for process heat. If the 8% not used for process heat was converted to ethanol, then one could expect an additional 2200 L of ethanol per hectare, bringing the ethanol yield per hectare to 8200 L and reducing the land use needs by 29%. If sugarcane trash were collected and used for energy and 50% of the bagasse used for ethanol production, this could generate an additional 3700–4000 L/ha ethanol (9700–10,000 L/ha total), and thus, reducing the land use requirement by a total of 33–38%.

Luo et al. (2009) presented a comparative study of life cycle assessment on gasoline and ethanol as fuels, and with two types of blends of gasoline with bioethanol, all used in a mid size car. The results of two cases were presented: (a) base case – bioethanol production from sugarcane and electricity generation from bagasse, and (b) future case – bioethanol production from both sugarcane and bagasse, and electricity generation from the wastes. In both the cases, sugar was co-produced. The life cycles of fuels included gasoline production, agricultural production of sugarcane, ethanol production, sugar and electricity co-production, blending ethanol with gasoline to produce E10 (10% of ethanol) and E85 (85%), and finally the use of gasoline, E10, E85 and pure ethanol. Furthermore, a life cycle costing was conducted to give an indication on fuel economy in both the cases. The results showed that in the base case, less greenhouse gas was emitted and the overall evaluation of these fuel options depended on the importance attached to different impacts. The future case is certainly more economically attractive, which has been the driving force for the development of ethanol industry in Brazil. Nevertheless, the outcomes depend very much on the assumed price for the crude oil. In the real market, the prices of fuels are very much dependent on the taxes and subsidies.

Technological development can surely help in lowering, both the environmental impact and the prices of the ethanol fuels. Intense research has been carried out for obtaining efficient fermentative organisms, low cost fermentation substrates, and optimal environmental conditions for fermentation to occur (Siqueira et al., 2008).

3. Feedstocks for the production of bioethanol: the case of sugarcane bagasse

In last 15 years, an increasing effort has been made towards a more efficient utilization of renewable agro-industrial residues, including sugarcane bagasse (Pandey et al., 2000; Baudel et al., 2005). According to Balat et al. (2008), bioethanol feedstocks can be conveniently classified into three types: (i) sucrose-containing feedstocks (e.g., sugar beet, sweet sorghum and sugar cane), (ii) starchy materials (e.g., wheat, corn, and barley), and (iii) lignocellulosic biomass (e.g., wood, straw, and grasses). The availability of feedstocks for bioethanol can vary considerably from season to season and depending up on geographic locations, could also pose difficulty in their availability. The changes in the price of feedstocks can highly affect the production costs of bioethanol (Yoosin and Sorapipatana, 2007). Because feedstocks typically account for greater than one-third of the production costs, maximizing bioethanol yield would be imperative (Dien et al., 2003).

Two-third of world sugar production is from sugarcane and one-third is from sugar beet (Linoj et al., 2006). These two are produced in geographically distinct regions. Sugar cane is grown in tropical and subtropical countries, while sugar beet is only grown in temperate climate countries. Since bioethanol trade is mainly from the South, feedstocks may eventually impact cane sugar trade (Balat et al., 2008). Both of these seem to be the most promising sources for bioethanol production (UNCTAD, 2006).

Other agricultural crop residues such as corn stover, wheat and rice straw, residues from citrus processing, coconut biomass, grasses and residues from the pulp and paper industry (paper mill sludge), and from the extraction of castor and sunflower oil as well as municipal cellulosic solid wastes, could eventually be used as raw materials to produce ethanol. However, each source of biomass represents a technological challenge. In the case of Brazil, there is no reason yet to explore other sources. The actual bioethanol system employees sugarcane efficiently and, in the next years, sugarcane bagasse will be used as lignocellulosic material with great success.

Brazil is the largest producer of sugarcane with 495 billion tons (Unica, 2009). The centre-south region of Brazil accounts for almost 80% of feedstock production (Zarrilli, 2006). The Brazilian bioethanol industry was poised for a major jump during 2006–2008 as a part of new national plan to increase the sugarcane production by 40% by 2009 (Renewable Energy Policy Network, 2006). Sugarcane bagasse (or, "bagasse" as it is generally called), is a porus residue of cane stalks left over after the crushing and extraction of the juice from the sugarcane (Pandey et al., 2000). It presents a great morphological heterogeneity and consists of fiber bundles and other structural elements such as vessels, parenchyma, and epithelial cells (Sanjuan et al., 2001). It is composed by 19–24% of lignin, 27–32% of hemicellulose, 32–44% of cellulose and 4.5–9.0% of ashes. The remainder is mostly lignin plus lesser amounts of minerals, waxes, and other compounds (Jacobsen and Wyman, 2002). Sugar mills generate approximately 270–280 kg of bagasse (50% moisture) per metric ton of sugarcane (Rodrigues et al., 2003). The Brazilian annual production of sugarcane bagasse is currently estimated at 186 million tons of bagasse.

The deployment of the sugarcane bagasse ethanol technology in Brazil is favored because the production process can be annexed to the sugar/ethanol units already in place, requiring lower investments, infrastructure, logistics and energy supply. Besides, the bagasse is generated at the industrial units, and as such free of transportation costs. This is a promising scenario because from each 10 million tons of dry biomass, 600 million gallons of ethanol could be produced, considering the use of its cellulosic part only.

4. Production of bioethanol from sugarcane bagasse

The production of fuel ethanol from lignocellulosic biomass includes biomass pre-treatment, cellulose hydrolysis, fermentation of hexoses, separation, effluent treatment, and, depending upon the feedstock, gathering, which may have an additional cost (Ojeda and Kafarov, 2009). Intensive efforts have been made in recent years to develop efficient technologies for the pre-treatment of bagasse, developments enzymes for enhanced cellulose/hemicelluloses saccharification and suitable technologies for the fermentation of both C_6 and C_5 sugars. Apart from the required basic research to support further developments in these fields, the solution for these challenges seems to be dependent upon an integrated and highly multidisciplinary approach as these research lines are indeed very strongly related.

4.1. Pre-treatment of sugarcane bagasse

Pre-treatment is one of the key unit operations for the successful conversion of lignocellulosic materials to ethanol. This is due to the close association that exists among the three main components of the plant cell wall (cellulose, hemicelluloses and lignin), which is by far the most determinant factor for the low accessibility of plant carbohydrates to biological processes such as enzymatic hydrolysis and fermentation (Gámez et al., 2006). Therefore, the main role of a pre-treatment method is to decrease the interaction between the main components of cell wall and make them susceptible to both saccharification and fermentation (Gámez et al., 2006). Likewise, the best pre-treatment conditions must be defined as those in which the maximum recovery of water-soluble hemicellulose sugars is obtained, along with the production of the best possible substrate for enzymatic hydrolysis and fermentation.

From an economic point of view, pre-treatment is a key step in the bioconversion process because it must improve the separation among cell wall components while avoiding the formation of compounds that are inhibitory to the subsequent hydrolysis and fermentation processes. Many methods have been used for pretreating lignocellulosic materials. These are steam explosion (Hernández-Salas et al., 2009; Hendriks and Zeeman, 2009; Balat et al., 2008; Ramos et al., 1992, 2000; Glasser and Wright, 1997), alkali washing (Hernández-Salas et al., 2009; Hendriks and Zeeman, 2009; Balat et al., 2008), lime, alkaline hydrogen peroxide, dilute acid hydrolysis (Hernández-Salas et al., 2009; Balat et al., 2008; Zhang et al., 2007), ammonia fiber explosion (Hendriks and Zeeman, 2009; Balat et al., 2008), liquid hot water and wet oxidation (Hendriks and Zeeman, 2009; Martín et al., 2008), among others. Each one of these methods has advantages and disadvantages and no one seems to be optimal for all practical applications involving different types of lignocellulosic materials.

Since the bioethanol program in Brazil has been primarily focused on sugarcane techniques for its pre-treatment have been extensively studied. Steam explosion is one the most widely used pre-treatment methods for fractionating the three main biomass components in different process streams (Martín et al., 2008; Balat et al., 2008). Steam explosion is a process whereby lignocellulosic materials are exposed to high pressure steam under optimal conditions, followed by quenching the reactor content to a pressure vessel (cyclone) by adiabatic expansion (Lee, 1997; Balat et al., 2008; Hendriks and Zeeman, 2009). This results in a substantial breakdown of the lignocellulosic structure, led by hydrolysis of the hemicellulosic fraction and depolymerization of cellulose and lignin. As a result, the susceptibility of plant polysaccharides to acid or enzymatic hydrolysis is greatly improved (Ramos et al., 1992; Excoffier et al., 1991; Balat et al., 2008; Ruiz et al., 2008; Martín et al., 2008; Hernández-Salas et al., 2009; Hendriks and Zeeman, 2009). Steam explosion also provides a significantly lower capital investment than many other pre-treatment methods, as well as a relatively lower environmental impact due to the use of less hazardous process chemicals (Ruiz et al., 2008). On the other hand, steam explosion produces a relatively low bulk density pretreated material that has to be washed prior to hydrolysis and fermentation to remove inhibitors such as phenolic acids and dehydration by-products derived from pentoses and hexoses (furfural and hydroxymethylfurfural, respectively) (Gámez et al., 2006). In fact, the versatility and wide application of steam explosion have given this technology a great compatibility with the concept of biorefineries, through which a variety of precursors and chemical inputs can be produced from renewable resources.

In Brazil, several research groups have been involved with the development or evaluation of different pre-treatment methods, such as organosol, auto-hydrolysis, steam explosion, acid hydrolysis, alkaline hydrogen peroxide and alkaline extraction, primarily. Alkaline washing of bagasse was shown to extract most of the lignin matrix and make cellulose and hemicelluloses more available to enzymatic hydrolysis (Pandey et al., 2000). Similar treatments were also useful to enhance the enzymatic hydrolysis of cane leaves (Hari-Krishna et al., 1998; Bhat, 2000).

4.2. Production of enzymes and enzymatic hydrolysis of bagasse

Although the pre-treatment is required to make the biomass accessible to the enzymes action, it is desirable to use mild conditions that minimize the degradation of the sugars and lignin into inhibitory by-products (Almeida et al., 2007). Therefore, to improve the enzymatic hydrolysis process and offsetting the low severity applied during the pre-treatment, the trend is the use enzyme mixtures containing xylanase and other accessory enzymes such as feruloyl esterase (Meyer et al., 2009). The use of these enzymes, naturally secreted by cellulolytic fungi, in the deconstruction of biomass has been considered an interesting approach.

The enzymatic hydrolysis can be carried out separately from the alcoholic fermentation, a process known as Separate Hydrolysis and Fermentation (SHF) or both processes can run together as Simultaneous Saccharification and Fermentation (SSF). In the SHF process, hydrolysis can be done at temperatures as high as 50 °C, taking advantage of enzymes stability at this temperature to increase rates and minimize bacterial contamination. It also allows easy separation of the sugar syrups from the hydrophobic lignin that can be used as solid fuel. Nevertheless, SHF leads to the accumulation of the glucose derived from the hydrolysis of cellulose that can inhibit the endo-and exo-glucanases and β-glucosidase, affecting the reaction rates and yields. As the subsequent fermentation step is run separately from the hydrolysis step the yeast cells can be recycled or used as animal feed, a usual and well regarded practice in the Brazilian ethanol industry.

In the SSF process the producing ethanol is faster, as the glucose formed is simultaneously fermented to ethanol. Besides, the risk of contamination is lower due to the presence of ethanol, the anaerobic conditions and the continuous withdrawal of glucose. The process also presents a lower cost as only one reactor is necessary. In this context, it is interesting to note that the ethanol that accumulates in the medium does not significantly affect the enzymes activity. The difficulty of this process relates to the different optimum temperature for enzymatic hydrolysis (45–50 °C) and alcoholic fermentation (28–35 °C).

Ethanol concentration is an important parameter to evaluate the feasibility of biomass ethanol. Fermentations of biomass syrups with 70 g/L glucose would reach a maximum of 35 g/L ethanol; well bellow the desirable minimum concentration. For comparison, the sugarcane juice presents 120–160 g/L sucrose that upon *Saccharomyces cerevisiae* fermentations results on ethanol concentrations in the range of 60–80 g/L. Considering that the biomass syrups, present glucose (C6) and xylose (C5) many efforts have been made to develop a transgenic microorganism able to co-ferment the glucose/xylose biomass syrups, with gain in ethanol yields. However the need to use a GMO would have an environmental downside and its use debatable as the ethanol resulting from xylose fermentation would be approximately half of that obtained from glucose (Stambuk et al., 2008). Since Brazil could chose the C6 sugars fermentation, the required concentration of fermentable sugars would be obtained by mixing the biomass syrups with sugarcane juice and/or molasses from the pre-existing plant and using the traditional yeast *S. cerevisiae* for ethanol production.

Brazil possesses an immense biodiversity which is believed to comprise 10–20% of the total known species on the earth. Many Brazilian groups are carrying out screening programs in order to isolate and identify microorganisms capable of producing lignocellulolytic enzymes.

Fungi are able to degrade cellulose, hemicellulose and lignin in decaying plants by a complex set of excreted hydrolytic and oxidative enzymes, such as cellulases, hemicellulases and ligninases, while filamentous bacteria mostly of the genus *Streptomyces* are able to degrade lignocellulose found in soil and composts also via the activity of the aforementioned enzymes. A large variety of microorganisms can degrade sugarcane bagasse including fungi from the genera *Trichoderma*, *Penicillium*, *Aspergillus* and *Humicola grisea* var *thermoidea*, whilst cellulolytic bacteria include the aerobic actinomycetes, the facultative anaerobic *Bacillus* and *Cellulomonas* and strict anaerobic *Clostridium*. The cellulolytic microorganisms produce a complex mixture of enzymes, which collectively have specificity for the β-1,4-glucosidic bonds. Depending on the site of action on the cellulose polymer, they have been classified as exo-glucanases/cellobiohydrolases (EC 3.2.1.91) and endoglucanases (EC 3.3.1.4). The cellobiohydrolases or CBH that, shows a higher affinity for crystalline cellulose, remove the disaccharide cellobiose from the reducing and the non-reducing ends of the cellulose chain. The endoglucanases or EG, with a higher affinity for the amorphous regions of the cellulose, promote a random internal attack in the cellulose chain, producing cello-oligosaccharides, which are substrate for the CBH enzymes. Cellobiose, that is water-soluble, is hydrolyzed into two molecules of glucose by the β-glucosidase (EC 3.2.1.21), also known as cellobiase (Zhang and Lynd, 2004). As the role of this enzyme is critical to complete the cellulose hydrolysis into glucose and also to avoid the accumulation of cellobiose, a potent inhibitor of cellulases, the level of cellobiase activity in cellulases preparations is critical.

Because many microorganisms secrete limiting amounts of enzymes, heterologous expression in other cell hosts has been employed to improve enzyme production. This has been the case of the xylanase from *Thermomyces lanuginosus* which was successfully expressed in the methylotrophic yeast *Pichia pastoris* (Damaso et al., 2003). This yeast presents several advantages over others systems for hyper-expression of enzymes and work has been done in order to develop improved vectors for this system.

An exciting and promising source of new lignocellulolytic enzymes is represented by the metagenome of non-cultivable microorganisms from different Brazilian biomes. In order to access this potential, several projects are being conducted in the Amazon, Atlantic Forest and Central Savanna.

5. Brazilian perspectives on bioethanol from sugarcane biomass

Presently, a revival of research on biomass ethanol is taking place in Brazil and some representative projects have been carried out. One such initiative is the "Bioethanol Production by Enzymatic Hydrolysis of Sugarcane Biomass". It aims at the development of an integrated technology for the conversion of sugarcane biomass (bagasse and straw) into fuel ethanol, including *on site* enzymes production. This project, which was initiated in 2006, is supported by the Brazilian Ministry of Science and Technology through the Research and Projects Financing (FINEP) and is carried out by a network of more than 20 institutions, including universities and research institutes. The project has its focus on: (i) screening and identification of cellulolytic and xylanolytic microorganisms from different Brazilian environments; (ii) strain optimization through mutation and using molecular biology; (iii) development of cellulases with higher specific activity; (iv) theoretical studies on the mechanism of action of the multi-enzyme complex; (v) enzyme production via submerged and solid-state fermentation; (vi) chemical and biochemical characterization of the enzymes; (vii) proteomics of fungal extracellular enzymes; (viii) engineering of enzyme blends; (ix) enzyme effectiveness in the hydrolysis differentially pretreated sugarcane biomass; (x) enzyme/biomass loading; (xi) hydrolysis kinetics; (xii) sugar yields; (xiii) development of bioprocesses for the production and concentration of biocatalysts; and (xiv) development of reactors for biomass hydrolysis and separation of hydrolysates. The research done so far has allowed significant advances on the engineering of enzyme blends that include cellulases, xylanases, β-glucosidase and accessory enzymes, customized to be used on sugarcane bagasse and leaves.

Another major project concerns the tasks assigned to the National Institute of Science and Technology in Bioethanol (NIST–Bioethanol), which is framing a series of strategic science actions in the country. NIST–Bioethanol's expectation is that the combination of biological and physical–chemical processes will make the process more efficient. This combined process has been named the third generation bioethanol that demands a larger input of research and technology. The fourth generation bioethanol is the one in which the plant itself (genetic modification of a characterized variety) will be genetically able to produce the necessary enzymes that participate in the process of digestion of its own cell wall. This is what is called auto-hydrolysis that should minimize the costs of bioethanol production. This will require that sugarcane is efficiently genetically transformed so that stable changes in its metabolisms, namely cell wall biosynthesis and/or hydrolysis, occur. NIST–Bioethanol is now defending the integration of the four generations of bioethanol so that their combinations could lead to a much more efficient, productive and sustainable bioenergy.

Besides the methods of hydrolysis of the wall, the progress in the knowledge on the physiology of plants used for the ethanol production, the employment of genetic tools and industrial engineering shall perform important roles in the increase of the ethanol productivity, independently of the "generation".

6. Conclusions and perspectives

Brazil has shown to the entire world an impressive energetic matrix with almost 44% of renewable resources. Great potentialities are observed for energy production from biomass. Brazilian bioethanol program is an example of the efficiency of sugarcane production and high technology bioethanol production. The Brazilian Government has recently published the Rule Project (PL 6.077/2009) that treats about the Agroecological Sugarcane Zoning (Zoneamento Agroecológico da Cana-de-açúcar – ZAE). This project aims limits the construction of new bioethanol plants or the enlargement of the cultivated area in about 50% of the Brazilian territory, including the Biomes Amazon and Pantanal. According to the Petrobrás Biocombustíveis, the bioethanol production in Brazil may triplicate till 2020, passing from the actual 27.5 billion liters to 70 billion liters. The production of sugarcane, which is detonated from bioethanol production, occupies only 0.9% of areas that can be cultivated (excluding the areas of environmental protection). For food production, 15.98% of cultivable land is used. Thus, Brazil has sufficient territorial space to raise significantly the production of food and, also, the biofuels. However, in the years to come, the increase in the Brazilian biofuels production will probably be strongly attached to the use of biomass (sugarcane bagasse and leaves), which will not necessary demand for new agricultural areas.

Acknowledgements

Authors thank MCT, CNPq, CAPES, FINEP, FAPESP and PETROBRAS for their support.

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