Marcos Silveira Buckeridge
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Routes to Cellulosic Ethanol

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Introduction

This book is a result of one of the workshops organized by the BioEn, the Bioenergy Program of the Foundation for Advancement of Science in the State of São Paulo (FAPESP). The BioEn was established in 2009 aiming to bring together the research in bioenergy at São Paulo State, which is the second largest producer of sugarcane in Brazil and one of the largest producers of bioethanol in the world in 2010.

This book is also a product of the National Institute of Science and Technology of Bioethanol (INCT-Bioetanol), presenting some of the results of its associated laboratories and collaborators.

Brazil and US are presently the largest producers of bioethanol on Earth and motivated by the growing effect of the global climatic changes and also energy security, both countries are focusing on increasing even more the production of this important liquid biofuel for economical reasons too.

The obvious way to do that using biomass feedstocks is to learn how to extract energy from the cell walls as they form up to 70% of the plant body. The valuable polymers composed of carbohydrates linked by glycosidic linkages are either left in the field for microorganisms to use them or are used for production of electricity (in the case of sugarcane in Brazil) in a not so efficient way.

There is a lot to learn and the biological sciences are now in an excellent position to provide valuable information that can lead us to potentially double the production of bioethanol.

However, reaching this goal is not a trivial task. As will be seen in the chapters of this book, the main targets are related to aspects concerning how to control the architecture of the plant cell walls by modifying plant genome for instance and at the same time to find microorganisms that are able to degrade the cell walls efficiently and produce free sugars that can be fermented by yeast. In order to do that, one needs to learn also about enzyme structure and how enzymes interact with carbohydrate substrates.

Microorganisms have the potential to be redesigned by molecular biology techniques and soon by synthetic biology, so that efficient enzyme cocktails can be produced and introduced commercially. Also, yeast will have to be taught how to use pentoses, along with hexoses, in order to produce ethanol.

The process of bioethanol production from biomass feedstocks such as maize, sugarcane and miscanthus, eucalyptus, and others will have to include also the agro-

nomical dimension of the problem that will have to be connected to the industrial processing. However, in this book the agronomical side of the story is not visited.

In this book, some chapters deal with bioenergy in general, comparing the energy matrices of US and Brazil and also comparing different forms to produce bioenergy, such as gasification, pyrolysis, and biodiesel from oils. However, the main focus is on different aspects that are important to reach better ways to deconstruct biomass, i.e., cell walls.

However, we did not forget to include information about the thermal route, because we believe that all means of science have to be applied in order to increase the production of renewable energy to cope with the enormous challenges that humanity is facing in this century.

We hope that this book will be a contribution to help this part of science and technology to advance.

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Part I
Bioenergy

Chapter 1
The Role of Biomass in the World's Energy System

Jose Goldemberg

1 Introduction

Since the dawn of civilization until the middle of the nineteenth century, biomass was the world's dominant source of energy and its consumption grew from approximately 50 million tons of oil equivalent in the beginning of the Christian era to 1,000 tons of oil equivalent today (a 20-fold increase). In this period, biomass has supplied the needs of the population for cooking and heating as well as shipbuilding, housing, and forges to process metals (mainly for weapons). Presently, biomass accounts for about 10% of the world's primary energy consumption. The other 90% is made up of nonrenewable fossil fuels (80%), hydroelectricity (2%), nuclear energy (6%), and renewable solar energies (2%) (Fig. 1).

The fraction of biomass used varies widely across different regions of the globe. It is as low as 3.9% in the OECD countries, 18.8% in all the developing countries as a whole, and it reaches 61.5% in sub-Saharan Africa (Table 1).

Such uses, in many cases, have led to a reduction of the forest cover of countries and regions of the world. This was pointed out as early as 400 BC by Plato when mourning the lost forests described by Homer that covered the barren hills of Greece centuries ago. As a whole, there was a reduction of 7.01 million square kilometers in total world's forest area since preagricultural times to the present, mostly for food production, although the contributions of energy use to such reduction are not negligible, particularly in Africa and Latin America and the Caribbean.

With the large increase in population since 1500 CA and particularly after the end of the eighteenth century with the development of the Watt machine, coal started to replace biomass. In the twentieth century, oil and gas entered the scene and contributed decisively to replace coal as well as biomass as can be seen in Fig. 2.

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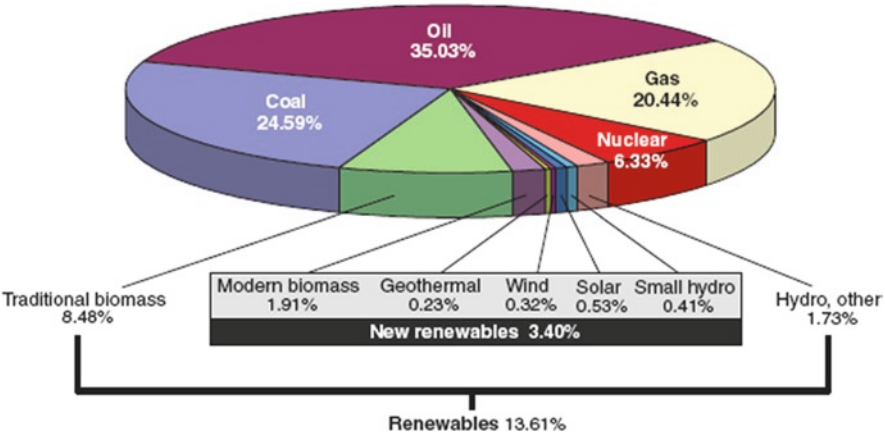

Fig. 1 World total primary energy supply 2004, shares of 11.2 billion tons of equivalent, or 470 EJ (Goldemberg 2007)

Box 1 Definition of Biomass (Goldemberg and Coelho 2004)

Biomass (plant matter) is usually classified into two categories:

i. "Traditional biomass," which is used in inefficient ways such as the highly pollutant primitive cooking stoves used by poor rural populations, leading to deforestation in many cases.
ii. "Modern biomass," which refers to biomass produced in a sustainable way and used for electricity generation, heat production, and transportation of liquid fuels. It includes wood and forest residues from reforestation and/or sustainable management as well as rural (animal and agricultural) and urban residues (including solid waste and liquid effluents).

The International Energy Agency (IEA) uses somewhat different definitions, "Combustible renewables and waste comprises solid biomass, liquid biomass, biogas, industrial waste and municipal waste. Biomass is defined as any plant matter used directly as fuel or converted into fuels (e.g., charcoal) or electricity and/or heat. Included here are wood, vegetal waste (including wood waste and crops used for energy production), ethanol, animal materials/wastes and sulphite lyes (...) also known as 'black liquor (...)'. Municipal wastes comprises wastes produced by residential, commercial and public service sectors that are collected by local authorities for disposal in a central location for the production of heat and/or power. Hospital waste is included in this category." The agency also recognizes that "Data under this heading are often based on small sample surveys or other incomplete information." The available statistics do not separate unsustainable sources of biomass

(continued)

Box 1 (continued)

(e.g., fuelwood from deforestation) from the sustainable (e.g., biodiesel). Until more comprehensive data are published for all countries, it could be assumed that all combustible renewables and waste (CRW) from developed countries are renewable; for developing countries, at least, the CRW applied into electricity production (thus a modern process) can also be considered renewable.

Source Renewable energy–traditional biomass vs. modern biomass" Goldemberg J. T. Coelho, Suani–Energy Policy 32 Nº 6 pp. 711-714, 2004

2 Energy and Transportation

The main reason for that was the fact that in the twentieth century, road transportation became one of the most significant consumers of oil products. Today, transport represents 22% of total energy consumption in industrialized countries and 14% in the developing countries. About half the world's oil production is consumed by road vehicles. The fleet's annual increase is about 10 million automobiles (doubling every 20 years or so) and five million buses and trucks worldwide (Goldemberg 1998). If the trend continues, a billion vehicles will use the world's roads by 2030. Not only is the number of automobiles growing but there is also a tendency to drive more, so the number of vehicle-miles traveled is increasing rapidly in countries such as the US.

The heavy dependence of transportation on oil is not a sustainable situation because of the problems associated with such resource:

1. Exhaustion of resources, which are estimated to last approximately 40 years with presently available technologies.
2. Security of supply, which is frequently threatened since most of oil used today comes from politically unstable regions (particularly the Middle East).
3. Environmental impacts, which can be local, regional, and global.

3 Environmental Impacts

Environmental impacts, particularly global ones, are presently becoming an overriding concern due to their impacts in climate change in contrast to local and regional impacts, which are already well known and being addressed by governments.

- *Local impacts* are mostly felt in cities such as Bangkok, Mexico City, Los Angeles, and Athens during peak traffic periods. At these times, air pollution in the city can approach crisis proportions and seriously affect the local population.

Table 1 Fractions of biomass in different regions of the world (2005) (International Energy Agency)

	Biomass						Total primary energy supply (TPES)	Share of biomass in World TPES
	Municipal waste[a]	Industrial waste	Primary solid biomass[b]	Biogas	Liquid biofuels	Total biomass		
	TJ	TJ	TJ	TJ	TJ	TJ	TJ	(%)
OCDE	832,261	271,863	6,142,592	376,773	725,782	8,349,271	232,266,749	1.77
Europe non-OCDE	8	6,588	254,458	390	0	261,444	4,396,935	0.06
Latin America	0	0	3,438,412	0	308,476	3,746,888	20,952,045	0.79
Asia	37,994	0	23,123,472	143,186	6,601	23,311,253	126,494,365	4.94
Africa	0	0	12,019,416	0	0	12,019,416	25,345,589	2.55
Middle East	0	0	43,052	0	0	43,052	21,073,714	0.01
Former USSR	315	149,204	352,462	569	328	502,878	41,036,418	0.11
World	870,578	427,655	45,373,856	520,918	1,041,187	48,234,194	471,565,815	10.23

[a] Municipal waste: the split for renewable and nonrenewable waste is also available

[b] Primary solid biomass: data are also available for charcoal

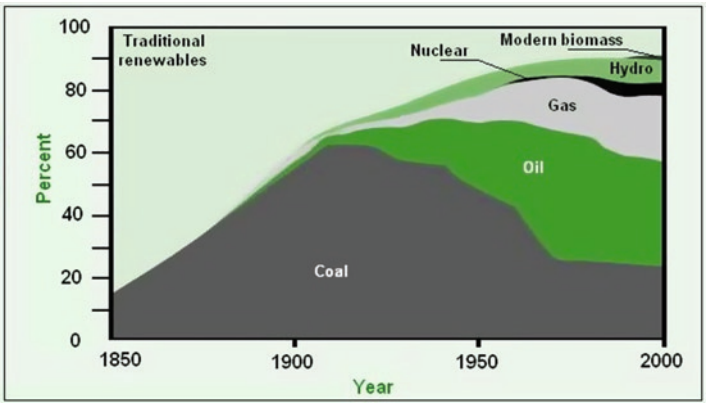

Fig. 2 (World Energy Assessment 2000)

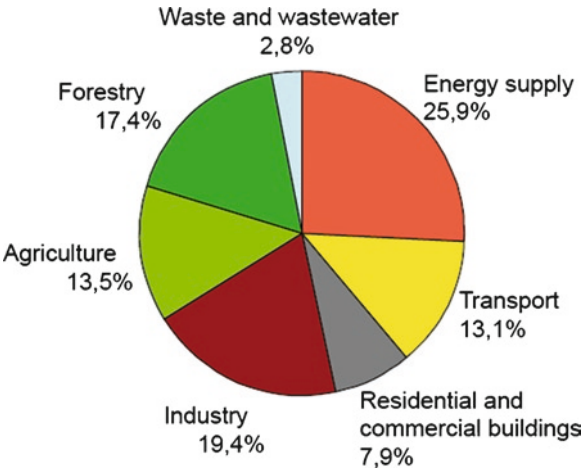

Fig. 3 GHG emission by sector in 2004 (IPCC 2007)

The problem is sometimes aggravated by a combination of local topographical and meteorological conditions that trap pollutants near the ground for extended periods of time.

- *Regional impacts* are mainly due to acid rain which is caused by nitrogen oxides emissions from the transport sector. The emissions from the increasing numbers of aircraft are estimated to total around three million tons annually (equivalent to about 15% of present automobile NOx emissions). In contrast to near ground-level emissions, where the nitrogen oxides are usually washed out by rain within days (generating acid rain), they persist in the upper atmosphere for long periods, contributing to ozone destruction.
- *Global impacts* are mainly due to the global fleet of motor vehicles, which is presently responsible for 13.1 of greenhouse gas emissions and 19.2% of the world's CO_2 output (Fig. 3).

4 Strategies to Face the Impacts of Transportation

There are three strategies to reduce the dependence of transportation sector oil:

1. Systems operation improvement
2. Technical approaches
3. Alternative fuels

- *System operation* includes shifting passengers and freight to transport modes that result in lower consumption and consequently lower emissions of pollutants and CO_2. Other measures include driving habits such as sharing and several restrictions on circulations of vehicles in problematic areas such as central portions of large cities as it was done in London.
- *Technical approaches* involve
    - *Engine efficiency improvement* – increasing effectiveness with which the fuel energy is converted into useful work for powering the automobile. Engine efficiency is the product of two factors: *Thermal efficiency*, expressing how much of the fuel energy is converted into work to drive the engine and vehicle and *Mechanical efficiency*, the fraction of work that is delivered by the engine to the vehicle.
- *Alternatives fuels* to gasoline for Otto-cycle automobiles and diesel for Diesel-cycle trucks
    - *Liquefied petroleum gas* (LPG) and compressed natural gas (CNG) have a higher hydrogen-to-carbon ratio than gasoline, thereby emitting less CO_2 per unit of energy. They have a higher octane number than gasoline, permitting the use of higher compression ratio engines. No major infrastructure changes are required for LPG or CNG use.
    - *Hydrogen* can fuel ultra-low-emission vehicles. Storage is a problem due to its low energy density. Compressed hydrogen storage is the most probable scheme, though liquid hydrogen or metal hydride storage is also possible.
    - *Biofuels* include ethanol produced from sugars and starch by fermentation with yeasts. Ethanol can be used pure or as a gasoline extender in spark-ignition engines. In addition, lignocellulose – from energy forestry, agricultural and forest industry residues, and the carbohydrate fraction of municipal solid waste (MSW) – is a further source of biomass liquids. Such a resource is 20 times more plentiful in the US than maize, and does not compete with food production (Fig. 4).

Box 2 Electrical Vehicles

Electric vehicles, using batteries, are of great interest today, especially as urban vehicles. If the electricity that fuels them comes from a nonfossil source, they can yield a significant greenhouse gas emission reduction. The key barrier to their implementation is the current state of chemical battery technology, resulting in high costs, heavy automobiles, and limited range. Also, while a gasoline automobile can be fueled in a few minutes, electric automobiles are generally fueled much more slowly over a time span of hours. Large-scale introduction of electric vehicles could require major infrastructure changes, not only in the energy distribution system and the automobile itself, but also in the electric power generation industry.

Fuel cells produce power electrochemically as opposed to combustion processes in conventional engines and can potentially reach significantly higher conversion efficiencies – perhaps by a factor of 2–3 – compared to today's internal combustion engine. Fuel cells come in several varieties, but the proton-exchange-membrane (also called solid polymer) fuel cell is the leading candidate for automobiles because of cost, size, simple design, and low temperature (>120°C) operation. The technology was originally used in the US space program. The fuel cells require hydrogen fuel, which may be generated on-board the automobile by reforming methanol or natural gas.

Source Inter Academy Council (2007)

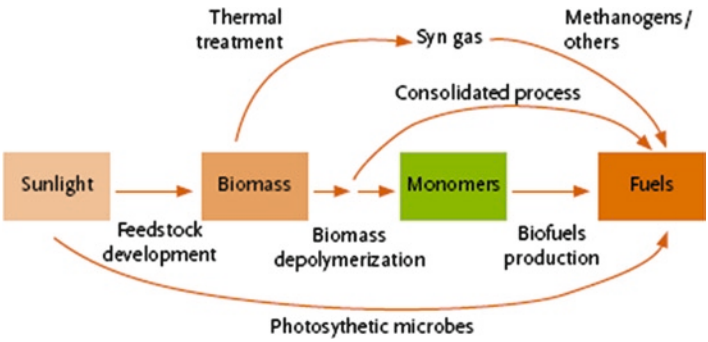

Fig. 4 (InterAcademy Council 2007)

5 Biodiesel and Ethanol

A number of plant-derived oils have also been considered for possible use as fuels in diesel engines including sunflower, soya, groundnut, cottonseed, rapeseed, palm oil, and castor oil. Vegetable oils have been tried unsuccessfully in the past, raising

problems of carbon deposits in the engine, clogged injection systems, high particulate emissions, reduced efficiency, and high maintenance needs. Diesel engines operating on these fuels have reduced efficiency and higher maintenance requirements.

Biodiesel oil is a potentially important enhancer or replacer of conventional diesel fuel. It can be prepared from many renewable raw materials that include soybean, rapeseed, and palm oils. The viscous, high-boiling triglycerides are processed to obtain more volatile methyl esters of the straight-chain fatty acids. Biodiesel oil is in the early stages of development, but specimens of it have undergone many successful long-term tests in buses, trucks, and tractors. In some of the tests, a mixture containing 80% conventional fuel and 20% biodiesel oil has been employed. Tests using 100% renewable fuel have also been successful. In both instances, the results were superior in many ways to those noted when conventional diesel fuel was employed. The renewable fuel is practically sulfur-free. It is nontoxic and quickly biodegradable if spilled. On combustion, it produces less toxic particulate matter. Only minor adjustments of existing engines are required to attain optimum performance.

Of all these approaches, the use of ethanol is the one that has reached maturity and is making a real contribution in reducing gasoline and diesel oil consumptions.

Production of ethanol to supply the needs of this fleet takes place in 405 distilleries, most of which are equipped for the dual production of sugar and ethanol. In 2007, production reached 22 billion liters. For 2008, the expected production was 26.1 billion liters and assuming a growth of 8% per year – which took place in the last few years – it should reach 30.5 billion liters in 2010 using approximately an area of four million hectares of sugarcane. There are at present 35 new distilleries starting production in 2008/2009 and another 43 in various degrees of implementation. In 2015, production should reach 47 billion liters and the land required approximately six million hectares (Goldemberg and Guardabassi 2008).

The cost of production of ethanol in Brazil dropped significantly over the years as seen in Fig. 5.

In 1980, it was roughly three times the price of gasoline in the international market, but it became competitive with gasoline in 2004 due to technological gains and economies of scale. Productivity increases of almost 4% per year in the last 30 years took place. The number of liters of ethanol per hectare of sugarcane increased from 3,000 liters per hectare to more than 6,000 liters per hectare. Ethanol is today fully competitive with gasoline without any subsidies (Goldemberg et al. 2004).

The drivers for such extraordinary expansion of ethanol production from sugarcane were not only economic and strategic – to reduce dependence from petroleum imports – but also environmental.

Ethanol does not have the impurities that come along with gasoline such as sulfur oxides and particulates, which are the main cause of the bad quality of the air in

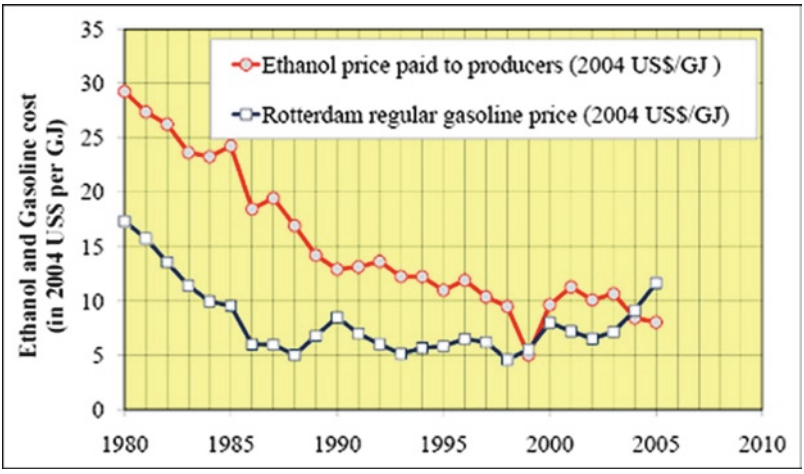

Fig. 5 The economic competitiveness of alcohol fuel compared to gasoline (Goldemberg et al. 2004)

large cities; examples are Beijing, Mexico city, São Paulo, and even Los Angeles. In the city of São Paulo, the quality of the air has improved remarkedly with the replacement of gasoline by ethanol, which today represents more than 50% of the fuel used by automobiles (CETESB 2008).

Emissions from land-use changes (including massive deforestation) could be a source of greenhouse gas emissions, as demonstrated by Fargione et al. (Fargione et al. 2008), but their study refers to a worst case scenario, which is not taking place presently, since expansion in the area used by biofuels is not taking place in virgin tropical forests. Such practice, of course, would release a large amount of CO_2, but extensive studies have been made on CO_2 releases, resulting from other agricultural practices that do not involve deforestation with results much less alarming.

There are almost 100 countries producing sugarcane in an area of 20 million hectares (approximately 0.5% of the world total area used for agriculture) (FAOSTAT 2007). The 15 most important producers representing 86% of total production of sugarcane. It is easy to convert plants producing sugar to ethanol distilleries, and most of the existing plants in Brazil have a dual purpose.

It is clear therefore that the production of ethanol from sugarcane could be expanded significantly if the example of Brazil is followed by several others using a fraction of the sugarcane for ethanol.

Ethanol can be produced from several feedstocks such as corn and other grains (mainly wheat), but the problem is the cost (Fig. 6).

Since the cost of production of ethanol from grains (in the US and Europe) is considerably higher than its cost of production from sugarcane (in Brazil); high import duties were imposed on ethanol imports in the US and Europe to protect local industries, which are therefore heavily subsidized. Table 2 gives estimates of

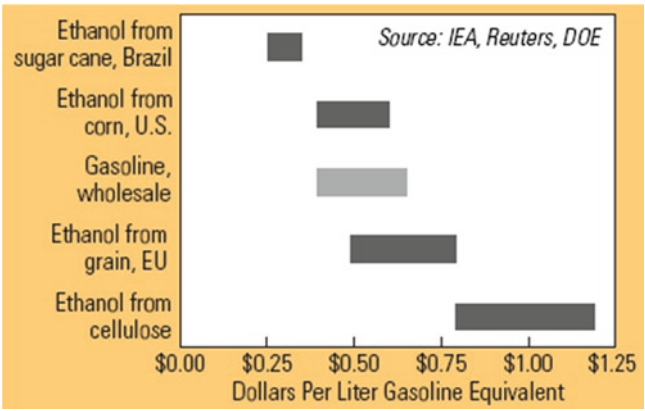

Fig. 6 Cost ranges for ethanol and gasoline production, 2006 (World Watch Institute 2006)

Table 2 Subsidies for biofuels in the US and EU 2006

	Ethanol			Biodiesel		
	Total billion	US$/liter	Billion liters	Total billion	US$/liter	Billion liters
Unites States	5.8	0.28	20.7	0.53	0.55	0.96
European Union	1.6	1.0	1.6	3.1	0.70	4.43
Total	7.4	–	22.3	3.63	–	5.39

the subsidies in the US and the European Union, which reached almost 12 billion dollars in 2006.

The removal of such subsidies is under discussion in the Doha round of negotiations, but prospects for progress in this area are not very good although countries such as France have decided to phase them out by 2012.

One of the reasons for the advantage of sugarcane is that all the energy needed for the processing comes from the bagasse which is not available using grains as the feedstocks. In this case, energy has to be "imported" by the distilleries most of which comes from fossil-derived fuels. This is the reason why the energy balance (i.e., the ratio of the energy contained in a litter of ethanol to the energy used in the process of preparation originating in fossil fuels) is 8:1 for sugarcane and 1.3:1 for corn. In a sense, ethanol from sugarcane is solar energy converted into a liquid while ethanol from corn is in reality fossil fuel (mainly coal in the US) converted into a liquid.

The consequence is that the greenhouse gas emissions resulting from the sugarcane route are much more favorable than from grains (Fig. 7).

6 Second Generation Technologies

However, progress in the use of cellulosic feedstocks of all kinds (including urban waste) using second generation technologies seems to be essential to broaden the feedstock used presently, which are in limited supply and could originate problems

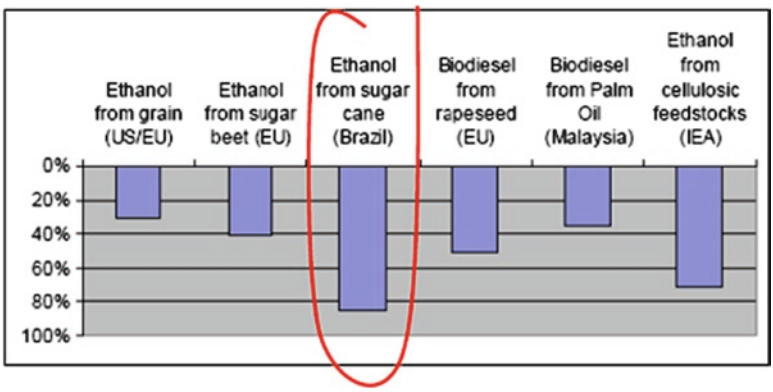

Fig. 7 Greenhouse gas reduction (Doornbosch and Steenblik 2007)

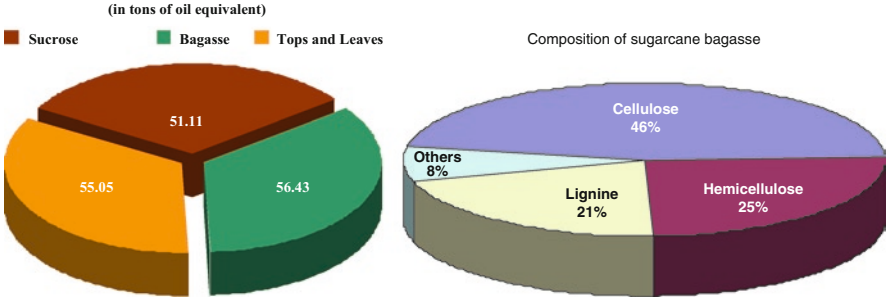

Fig. 8 Energy contained in 1,000 tons of sugarcane

such as a competition between fuels "versus" food. Excellent candidates for such feedstock are the bagasse of sugarcane and switchgrass.

In the case of sugarcane, bagasse contains a third of the energy contained in sugarcane, tags and leaves another third. With mechanized harvesting, which is progressing rapidly in Brazil, the available amount of such materials is increasing and is thus a prime candidate for second generation (Fig. 8).

Switch grass in the US seems to be an interesting option for the cellulosic route since it has a composition rather similar to bagasse.

Second-generation technologies that will allow the use of any cellulosic material for the production of biofuels are being actively pursued but have not yet reached commercial production.

This area is therefore open to new and creative approaches of great scientific technological and economic significance in the direction of replacing fossil fuels by renewable resources.

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Chapter 2
Bioenergy and the Sustainable Revolution

Wanderley D. dos Santos, Edgardo O. Gómez, and Marcos S. Buckeridge

1 Introduction

In this chapter, we will discuss some unexpected consequences that renewable energy policies might present for technological development and present an overview about the main current approaches to produce Biofuels. The technological barriers and alternatives investigated to overcome them are also discussed. In the first section, we argue that such radical changes in the way we think and sustain our development might imply that we are facing a new revolution in our energy production system. We proceed to elucidate some principles that are likely to determine the ideal and actual scenario of renewable fuels, including how ethanol can succeed and how biotechnological approaches chosen to produce second generation ethanol imply coping with the high complexity of lignocellulosic material. We also discuss the principles of biodiesel production, the importance of this incipient biofuel might offer to the setting of ethanol industry. Finally, we discuss the advantages and main perspectives in the short-term developments expected by the promising area of themochemistry to biofuel production.

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2 Energy Revolution

2.1 Mitochondrial Revolution

Evolution does not always occur as a soft continuum of myriads of little adaptations. It sometimes jumps. Since the origin of life around four billion years ago, green–blue bacteria increased the amount of molecular oxygen (O_2) in the atmosphere conspicuously. For most of the living organisms at that time (exclusively bacteria), oxygen was very dangerous. For some organisms it was (and still is) deadly. Thus, most bacteria lived only in oxygen-free environments. In the absence of oxygen, one of the main forms that heterotrophic organisms used to obtain energy was via the fermentation process. This process preserves part of the free energy content from a molecule of glucose in two adenosine-triphosphate (ATP) molecules, the standard energy fuel in catabolic processes. However, in this process, most of the chemical energy present in glucose is wasted as fermentation residues such as alcohol or lactic acid.

In the presence of oxygen, some organisms are able to accomplish cell respiration, a process in which glucose is completely oxidized to CO_2 and 36 ATP are produced from every single glucose molecule!. About two billion years ago, microorganisms undergoing selective pressure in an atmosphere that was becoming increasingly toxic with oxygen, developed the ability to tolerate and even obtain benefits from it. The last enzyme from the citric acid cycle to emerge and make possible respiration as we know it today was the α-cetoglutarate complex. This is thought to have occurred by mutations of genes of the pyruvate dehydrogenase complex, an enzyme complex with a similar structure and role in the citric acid cycle in aerobic as well as in anaerobic organisms (Fig. 1).

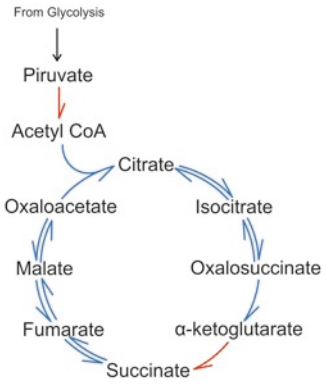

Fig. 1 Some anaerobic bacteria have the enzymes to produce several metabolic intermediates of the citric acid cycle. However, they cannot complete the cycle because they do not have the alpha-ketoglutarate dehydrogenase, which converts alpha-cetotarate into succinl-CoA (red arrow in the cycle). This enzyme probably evolved from pyruvate dehydrogenase. Such complex performs a similar reaction converting pyruvate into acetyl-CoA (red arrow above). Both complexes exhibit three analogous enzymes and use the same cofactors (TPP, lipoate, FAD, NAD, and coenzyme A).

This highly efficient novel system for using energy from carbohydrates completely changed the biological scenario, making a whole new level of complexity in living organisms possible. From that time on, with more available energy, multicellularity became viable and many other types of organisms evolved. In other words, this energetic revolution was so powerful that today, most unicellular eukaryotes, all fungi, animals, and plants are dependent on mitochondrial machinery, the organelle responsible for accomplishing cell respiration.

2.2 Modern Bioenergy: A Sustainable Revolution

With the increase in population, which is expected to reach over nine billion people by the year 2050, the issue of availability of energy appears to be crucial. Humans are increasingly better at improving health and elongating life span. Furthermore, capitalism requires profit, continuous production of all types of products in order to survive. Therefore, over the next 40 years, humans will need to find a way to greatly increase efficiency of energy production.

The problem is that in the cycle of energy production that we are in, which is essentially based on fossil fuels, the excess of production of some useful or even essential molecules residues such as CO_2 turned them into pollutants. As a result, we discovered that we were poisoning the atmosphere and changing the climate (IPCC 2007). The environmental impacts are now regarded as fundamental for the survival of humans on this planet. As a consequence, the production of energy, food, and all products consumed by human societies will need to come out from sustainable ways of production.

As the evolutionary burst supported by the mitochondrial energetic efficiency, we also experienced successful development cycles based on the exploration of coal throughout the Industrial Revolution and still enjoy, sponsored by oil. We need to find a way to produce more energy in order to supply our development aims for the decades to come. However, we know that the consequences of this step can cause problems further into the future, our choices must be based on approaches capable of making the energy production system progressively sustainable. Although there are nuclear and geothermal nonbiological ways to obtain energy, the use of biomass to produce energy is certainly one of the more realist ways to increase energy production in short term, especially thanks to the advances obtained during the twentieth century in the areas of biochemistry and molecular biology. The production of energy from biomass is not new as humans have been burning wood for thousands of years. However, we are now reaching a point in which we can think and design, through synthetic biology, forms to improve plant photosynthesis and cell wall architecture to make cell wall carbohydrate more accessible to hydrolysis and available for fermentation. These two targets can possibly work to significantly increase energy production. This is because (1) improving photosynthesis efficiency can increase productivity of biomass, and (2) gaining access to the monosaccharides of the cell walls opens

the way to obtain energy from ca 70% of the plant body thus greatly increasing the production of energy in the form of biofuels as ethanol, for instance. Most chapters in this book are about the biochemical routes to obtain lignocellulosic ethanol. However, it is not (and cannot be) the only one to be adopted in biofuel production. Transesterification of plant fat acids are used to produce biodiesel and it is thought that the production of such hydrocarbons as well as a wide variety of other chemicals is also possible by chemical routes such as pyrolysis and gasification, as we discuss later in this chapter. Whatever the route, the development of technologies to efficiently use renewable sources of energy might imply a new age of social development without the ghosts of global warming and petroleum shortage. Although such technologies alone do not mean an energy revolution, they can be thought as a radical change in ways humans understand economy and development. Production and goods do not move in closed mechanical cycles as taught by classical economy. Rather, civilization is an opened thermodynamic system in which crude matter and energy are continuously appropriated from nature to produce humans goods and residues and entropy are unavoidably produced (Cechin 2008). Being part of nature, we need to learn how to cope with nature's limits to supply our demands and recycle our residues. Using bioenergy in a sustainable way is currently the most realistic form to harmonize our ambitions for economic growth with the planetary constrictions. It is certain that some day we will find the limits of using renewables as well, given our boundless obstinacy for progress. Meanwhile, however, we seem to be starting a promising cycle of sustainable technological growth based in renewable sources of energy: the sustainable revolution.

3 Choices for Renewable Fuels

According to the second law of thermodynamics, in a chemical reaction the products will conserve a fraction of the existent energy in its reactants. In general, the energy potential of the products is lower than the reactants that made them. In this way, the amount of energy conserved by a molecule is inversely proportional to the number of chemical reactions necessary to build it. In this sense, perhaps the cheapest renewable fuel that we can produce is the molecular hydrogen (H_2). A source of energy (i.e., light, electricity, etc.) can be used to liberate hydrogen directly from water. As this process is direct (i.e., without many chemical steps) the efficiency of the conservation of the energy for the production of H_2 is relatively high. That type of direct production can also be accomplished with other molecules, but considering the amount of water on the planet, in practice, any other molecules are far less abundant. Another advantage of using H_2 is that its combustion produces no pollution, only water. However, although the production of H_2 can be cheap and clean, its use is not easy.

H_2 is a highly explosive gas and must be transported under pressure, which considerably increases the cost of transport and risks of accidents. From this point of view, liquid fuels seem to be a more convenient option for use in vehicles.

When compared with H_2, liquid fuels can present a considerable difference in energy density, related to difference in the oxidation state of carbon. Alcohols such as methanol and ethanol are more oxidized than lipids in the form of biodiesel and in this way, alcohols release less energy during combustion per unit of mass. The complete combustion of one gram of ethanol liberates about 7 kcal of energy while one gram of lipids releases ca. 9 kcal. Because H_2 is a gas, it presents an even smaller energy density. On the other hand, as discussed above, the fact that liquid fuels are more complex than H_2 implies that they are less efficient in energy conservation.

The processes involved in the synthesis of liquid biofuels such as ethanol is indirect and imposes a higher cost to obtain it. Part of the energy present in the sugars used to produce ethanol, for instance, encloses energy that had to be used for agricultural processes, planting, irrigation, fertilization, harvesting, plant transportation, milling, and industrial processes such as fermentation by yeasts, distillation, and subsequently fuel distribution. Therefore, in order to rationally choose an ideal fuel and production technology, one must consider the energy efficiency throughout the whole chain of production, consumption. and renewability.

3.1 Biodiesel from Plant Sources

Following the wave of ethanol success, other kinds of biofuels are now being developed as well in scale production, as biodiesel. In Brazil, plants such as palm, soybean, and other edible cultures are being partially used to produce biodiesel. On the other hand, nonedible plants able to grow in marginal lands and climates as semiarid and cerrado (Brazilian savanna) have been studied in order to avoid competition with traditional agriculture and food production. They have been studied and selected by their seed and seed oil yields, oil profiles, and rusticity. Such characteristics are found in many *Euphorbiaceae* species as *Jatropha curcas*, sea almond (*Terminlia catappa*), neem (*Azadirachta indica*). *J. curcas* and other genera have been considered as plants with the strongest potential for biodiesel production in Brazil with financial support for farmers from cerrado regions and industrial plants being build.

Oleaginous plant seeds store oil in cell structures called oil bodies. Seedlings use their reserve compounds as a source of carbon and energy until being able to self sustain. The principal lipids stored by oleaginous are triglycerides. They exist in esters of a residue of glycerol, a trihydroxylic alcohol known commercially as glycerin, with fatty acids. Fatty acids are interesting as fuel because of their high energy content.

However, the viscosity of triglycerides can be too high for its direct use in diesel engines. Therefore, they must to be converted to ethyl or methyl esters of fat acids in order to be useful. The transesterification process includes substituting the glycerol by ethanol or methanol using a chemical catalysts such as H_2SO_4, NaOH, KOH, or $NaOCH_3$. The length of fatty acids also influences the viscosity and energy density of biodiesel. The longer the length of the aliphatic chain, the higher the energy density, but the lower the viscosity will be. On the other hand, fatty acids might

present unsaturations, i.e., some carbons of the aliphatic chain might be oxidized to form one or more double bonds. These unsaturations produce breaks in the linear geometry of saturated (nonoxidized) fatty acid, decreasing the spatial proximity among molecules and as a consequence increasing its viscosity. In this way, the kind of alcohol residue esterified to fatty acids, the length of aliphatic chain and the degree of unsaturation of fatty acid residues are features that imply a trade off among viscosity and energy density for biodiesel. Thus, the fatty acid profile of different plants strongly determines the choice of plant species for biodiesel production.

3.2 Bioethanol

Ethanol is an organic compound used as liquid fuel in light vehicles since the invention of internal combustion motors by Nikolaus Otto. Today, it is the first renewable fuel produced from plants such as sugarcane in Brazil and corn in the USA. Carbon dioxide produced by burning ethanol is assimilated by plants from the air. Thus, ethanol does not generate a net unbalance of greenhouse gases as do gasoline, diesel, and other petroleum derivatives.

Currently considered a traditional producer of sugarcane, Brazil inaugurated the industry of ethanol for fuel applications early in the twentieth century. In 1973, an unprecedented increase in the price of petroleum harnessed to Yom Kippur's war and the seizure of the USA and western Europe by Middle East petroleum producers. As a result, the Brazilian government decided to increase the production of ethanol throughout an extensive program of incentives.

Currently, Brazil has no pure gasoline in any gas station. Flex fuel engines afford the choice to drivers to use E25 to E100 gasohol (25–100% of ethanol). Strategic concerns about energy security and global warming has impelled other countries to develop their own production of ethanol and in spite of the greater productivity of sugarcane, 2,105 gallons per acre against 495 gallons per acre of corn ethanol, the US overcame Brazilian ethanol production in 2006 and are today the largest producer of ethanol in the world.

However, the current means of production are far from being able to supply ethanol to support potential demand to the whole world. In this sense, governments and researchers have been driving their attention to explore the wide energy availability of lignocellulosic biomass in order to produce more biofuels, and do so more efficiently and sustainably. Currently, most of the biomass from sugarcane and corn is wasted as residue or inefficiently burned to run the mills. However, such biomass is formed mainly by sugars such as cellulose and other related polysaccharides. The problem is that they are linked to each other in complex ways, forming an interwoven network of polymers, which are very difficult to disentangle. However, once broken into free sugars these polysaccharides might be fermented to produce ethanol.

Two thirds of the energy produced by sugarcane is in the lignocellulosic material. Besides being the most abundant biomolecules in nature, technologies able to hydrolyze holocellulose (cellulose and hemicelluloses) in its monosaccharides at a low cost, will

make possible the utilization of the most diverse plant residues for production of ethanol. Such technologies might, in theory, double bioethanol productivity, thus helping to avoid the expansion in the area needed to produce biofuels and consequently avoiding impacts on forests by indirect land use.

3.3 Biochemical Conversion

The biochemical approach toward saccharification of lignocellulose biomass is based on the principle that catalysts may decrease the activation energy and accelerate the velocity of the hydrolytic reaction. A small amount of a specific catalyst might accomplish a number of reactions virtually infinite. Due to the complexity of lignocellulosic material, a cocktail of enzymes will have to act in concerted fashion in order to carry out the hydrolysis of the great number of reactions necessary to release all monosaccharides present. It would need to cope with the lignin present in the mixture and also with the crystalline (water free) cellulose, which is resistant to most of the physical and chemical attacks (Soccol et al. 2010).

In order to overcome these barriers, biomass has to be prepared beforehand and this process is called pretreatment. Several types of pretreatments have been made. They consist of methods able to increase the surface area of polysaccharidases available to enzyme attack. In thermo acid treatment, lignin is partially removed, exposing polysaccharides to enzyme hydrolysis. Alkali treatment may also be used to remove ester linkage between lignin along with pectic and hemicellulosic polysaccharides. Indeed, polysaccharides might be dissolved by strong alkali and hydrolyzed by mild acid treatments with sulfuric acid. However, once the glycosidic linkages are broken, the monosaccharides might be easily oxidized to furfurals and hydroxymethylfurfurals. The different degrees of susceptibility from α and β linkages, as well as the pectin connection among fibers (middle lamella), results in oxidation of significant parcels of the carbohydrates. Pentoses, furfural derivatives, and phenylpropanoids from lignin will inhibit subsequent sugars' fermentation to ethanol by yeasts, reducing the efficiency of direct chemical hydrolysis. Ethanol at high temperature might be used to partially extract lignin and other soluble solids in organosolv® process developed by the Dedini Co (Ramires et al., 2010). These and other processes are used in the paper industry and have been adapted as pretreatments for ethanol production from lignocellulosic material. Other processes have been developed specifically for bioethanol technology. One of such processes is steam explosion, a method in which biomass is submitted to high pressure and left to expand fast in presence of vapor exposing the fibers to subsequent hydrolysis.

The biochemical route is in fact an application of modern techniques of cell and molecular biology (Buckeridge et al. 2010). The possibilities are many (Fig. 2). Bioenergy feedstock species can be genetically modified so that their cell walls become more accessible to enzyme hydrolysis during the industrial process. The microorganisms that will be used to produce the enzymes that will perform hydrolysis can be engineered, and the ones that will ferment the sugars, which in the case of

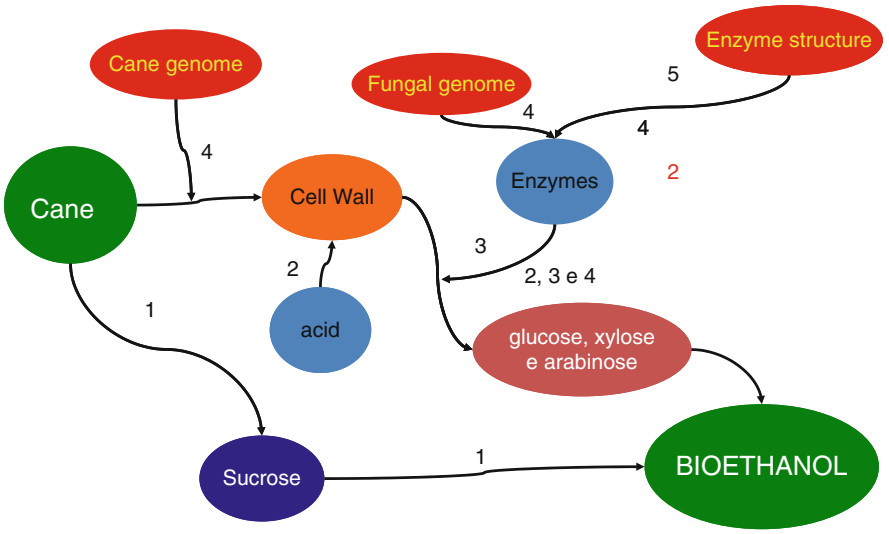

Fig. 2 Strategies for the biochemical route to obtain cellulosic bioethanol from sugarcane. Numbers refer to phases that will need to be followed in time, in order to complete the process of cellulosic ethanol. Phase 1 is already completed, but must be improved; it is called first generation. Phase 2 refers to the use of acid hydrolysis couple with enzyme hydrolysis to produce fermentable sugars. During phase 3, enzyme cocktails will be available and enzymatic hydrolysis will be mastered in large scale. On phase 4, modified walls, better enzyme producing fungyi, and modified enzyme structures could be used to improve the industrial process even more (from Buckeridge and Salatino, 2010)

grasses are pentoses, will need to have their metabolism adapted to use this kind of sugar. In the era of synthetic biology, the biochemical route will probably join other industrial processes in a revolution without precedents in biology, i.e., industrial processes strongly based on biological mechanisms.

3.4 Thermochemical Conversion

Synthesis gas generated from catalytic reform of fossil fuels (natural gas), or gasification of coal, is a versatile platform in conventional chemical and energy industries. By thermochemical processes, lignocellulosic biomass can be converted into biofuels and other derivatives (Fig. 3). The main advantages of such an approach do not cope directly with the natural complexity of biomass as in biochemical approaches and the low intensity of pretreatment involved. The cores of thermochemical conversion are the processes of pyrolysis and gasification. Pyrolysis is a heating procedure performed in complete absence of oxygen. It produces different phases depending basically on parameters such as temperature, pressure, time of reaction, and heating rate. Among the phases, one is an oil (bio-oil) that can be used to feed the gasification process. Gasification can be accomplished with a controlled amount of oxygen and is driven toward obtaining a product gas, also named synthesis

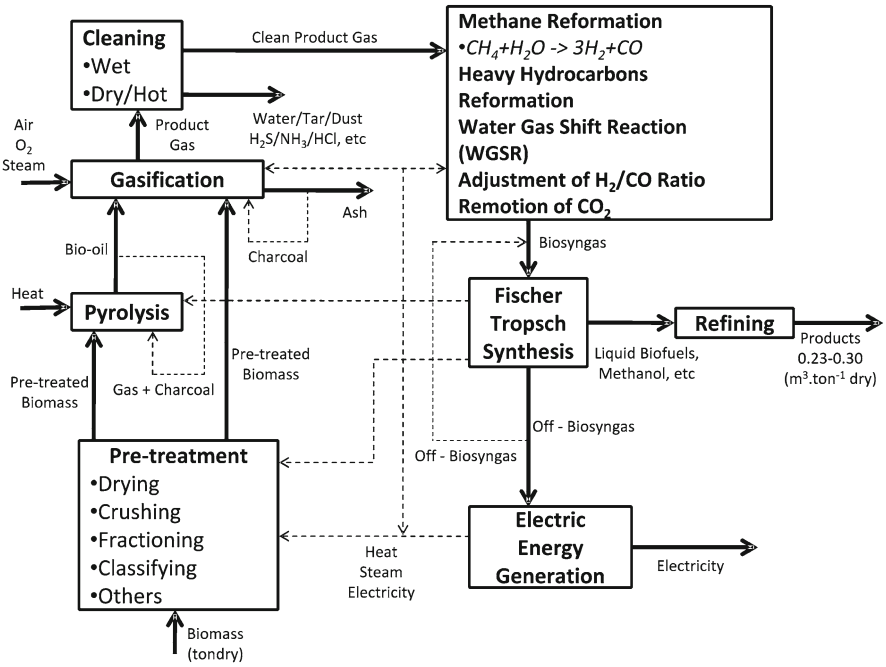

Fig. 3 Steps in thermochemical production of biofuel. The main thermochemical processes are pyrolysis and gasification. Both pyrolysis and the gasification produce different phases depending basically on temperature and pressure, reaction time, process time, heating rate, etc. The bio-oil from pyrolysis can be used to feed the gasification process. The biofuel synthesis processes have several requirements in relation to reactor temperature and pressure, type of catalysts, H2/CO ratio in syngas, which can be adjusted by water gas shift reaction (WGS) or by membrane separation techniques, impurities content in the syngas such as CO2, dust, tar, H2S, NH3, HCl, CH4, halogen and alkali compounds, and type of downstream processes. There have been propositions of polygeneration where integrated processes are used to produce syngas to chemicals, biofuels and generation electricity in the same plant and from the same feedstock

gas or syngas (Fig. 3). Syngas can be converted by fermentation or catalytic synthesis in liquid and gaseous fuels such as gasoline, diesel, ethanol, methanol, methane, and hydrogen, among other energetic and nonenergetic products (Rezaiyan and Cheremisinoff 2005; Knoef et al. 2005).

Theoretically, a syngas is composed of equimolar amounts of hydrogen (H_2) and carbon monoxide (CO), which goes through water gas shift reaction (WGS) and a further process to remove carbon dioxide (CO_2). Synthesis gas produced by direct gasification of lignocellulose or bio-oil is composed of solid and liquid particles (dust and tar), halogen and alkali compounds with inorganic impurities being hydrogen sulfide (H_2S), ammonium (NH_3), hydrogen chloride (HCl), methane (CH_4), and other light hydrocarbons (C_2H_6) which contaminate the catalysts used in downstream processes (Obando et al. 2010). There have been propositions of polygeneration in which integrated processes are used to produce syngas and generation of electricity in the same plant. Others consider the possibility to

integrate the biochemical with the thermochemical route by using by-products of the pretreatment processes.

3.5 Comparison of Thermochemical and Biochemical Routes

Thermochemical conversion is at a stage of development and evaluation on a pilot scale to improve the quality of the syngas as well as the capabilities needed to achieve economical viability. On the other hand, the biochemical route is presently at the precommercial stage of development due the great number of plants being implanted and already in operation. The current estimated costs of biofuel production by thermochemical route are around 0.5–0.6 U$ per liter of equivalent fuel, while the estimated cost of biochemical conversion is 0.7–0.9 US$ per liter of equivalent fuel. However, biochemical conversion must undergo a rapid cost reduction in the face of commercial plants being set until 2012, when costs are expected to reach about 0.3 US$ per liter of equivalent fuel (Lora et al. 2007).

Biochemical conversion presents great challenges in bioengineering of enzymes and yeasts, detoxification of substrates (pretreatment), as well as the energetic optimization and integration of the processes. Of course both routes require several unitary operations as harvest, transport, storage and final arrangement of biomass such as drying, fractionating and classifying of particles, to name but a few. But there are differences. Pretreatment, for instance, has a high impact on the cost of biofuels in both routes. However, pretreatments are considered of lower intensity in thermochemical approaches, when compared with pretreatment intensities required to biochemical conversion. Biochemical approaches demand improvement of cellulose accessibility to enzymes that are capable of hydrolyzing polysaccharides. This confers a considerable impact on the energetic balance of bioconversion.

Both technological platforms require large scale plants to reach economic viability. However, reported data suggest facilities to process 100 ton/h of dry biomass to biochemical plants are feasible, while about 500 ton/h of dry matter is necessary in order for a themochemical platform to become economically viable. In this last case, gasifiers of 150 ton/h of dry matter will be needed. This is currently a relevant technical and economical constriction for the thermochemical route. One promising study, although still on a laboratory scale, proposes the production of bio-oil by fast pyrolysis and successive gasification of bio-oil. If such technology becomes possible and safe, reactors able to process about 2 tons of biomass per hour could produce bio-oil in a decentralized way, centralizing the gasification an economically viable scale (Rocha 2008).

4 Concluding Remarks

The availability of useful types of energy strongly determines the evolutionary potential in nature. Human technological development is also highly dependent on energy availability. In recent history, coal and petroleum played an important role

in industrial and postindustrial revolutions. The pressure against environmental imbalance caused by greenhouse gases emissions due to the use of fossil fuels implies a strong barrier against the maintenance of the rates of social and economical development on the basis of oil and coal as main sources of energy. On the other hand, the potential to produce energy from biomass and other renewable sources exceeds (several times) the world current demand. The development of technologies to extract energy from renewable sources (such as ligonocelluloses) is the way to enter a new age of technological development.

Lignocellulose is the most abundant biological crude matter on the planet and is composed of high energy molecules. However, it is also a highly ordered cell structure which renders mechanical and biochemical resistance to plant tissues. Furthermore, cell walls present a relative high diversity among different plant species and might become progressively recalcitrant when it is (wrongly) disassembled. Nonetheless, several biological systems have coevolved with plant cell walls and optimized the biochemical conversion of cellulosic biomass using a similarly complex set of enzymes. We are now able to face this challenge using and advancing the knowledge about plant cell wall architecture. Because most species chosen as feedstocks for bioenergy are grasses, the primary focus of technological development will be the type II cell wall (i.e., the wall typical of grasses that is composed of arabinoxylans and mixed linkage glucans as main hemicelluloses). Although they represent just a small fraction of the plant species, grasses respond for ca. one-fifth of world green cover and more than four-fifths of food consumed by humanity, including forage and biofuel.

Some exciting synergy might be found between biodiesel and bioethanol production. Glycerol, a by-product of the biodiesel industry, might be fermented by yeast to produce ethanol. Ethanol, in turn, might be used to transesterify triglycerides and reduce viscosity. On the other hand, harvesters, tractors, and trucks used in the cultivation and transport of ethanol production and consumption chain today, all run on diesel engines, which negatively impacts sustainability of ethanol. Therefore, the emergence of large scale biodiesel industry might mean a snap point in sustainability of the whole chain of biofuel production.

There is an aspect toward the technological routes convergence. They offer opportunities for scientific development in areas such as development of new pyrolysis and gasification processes, catalysis applied to syngas production and purification, development of new pretreatments of biomass, enzymes and microorganism engineering, as well as energetic optimization and integration of the processes. All present potentials to be part of the solution and research in these areas must be put forward in order to guarantee that a better solution to the problem will be found in the shortest possible time and will be strongly based on high quality science.

Is seems, therefore, that humanity is living one of these moments of revolution in which the system will jump to a superior level of organization that will made us capable of going far beyond where we have been during the last centuries.

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Chapter 3
Biomass Gasification for Ethanol Production

Luiz A.H. Nogueira, Joaquim E.A. Seabra, and Isaías C. Macedo

1 Introduction

For a sustainable future, it is essential for mankind to access the largely untapped solar resource by innovative bioenergy routes, an important way to overcome fossil fuel dependence and mitigate related environmental impacts. In this framework, as a good example of the potential to be exploited, among the several biomasses under scrutiny to be used for energy supply, sugarcane appears as one with the most interest and potential, with estimates that about 142 million hectares currently are available for such culture, taking into consideration rain feed areas in tropical countries and without significant impact on food production and the environment (Fischer et al. 2008).

Sugarcane is a very productive plant, in Brazil and in many other wet tropical countries where it is cultivated, it averages annual yields of approximately 110 tons/ha (including stalks, tops and leaves), which translates to more than 35 tons of dry solid biomass, corresponding to an output of 110 barrels of oil equivalent to a hectare per year, produced with solar energy, water, carbon dioxide, and a reduced demand of exogenous energy in agricultural and transportation activities (Macedo 2005). This biomass, mostly polysaccharides (sucrose, cellulose and hemicellulose), is used as raw material for a large range of products such as table sugar, sugar syrups (glucose) and other sugar derivatives, food and feed additives, plastics, electricity, agrochemicals, and ethanol. An assessment conducted in 2005 identified more than 60 different technologies in the Brazilian industry currently using sugarcane as raw material (IEL/SEBRAE 2005).

Indeed, in the huge expansion of the Brazilian sugarcane agro industry in the last decades, it is remarkable to observe the development of a diversified biotechnology and chemical industry based on sugarcane products. However, the lignocelullosic by-product of sugarcane crushing, bagasse, a fibrous material composed by cellulose (40–60% of dry matter), hemicellulose (20–40% of dry matter), and lignin (10–25% of dry matter) remains essentially used only as fuel, burned in boilers to produce

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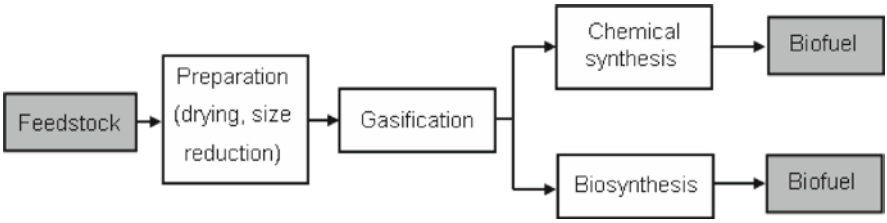

Fig. 1 Basic routes for biofuel production by biomass gasification

high pressure steam used in cogeneration schemes to supply heat and power to sugar mills and to the grid. In some mills where improvements in energy management have been introduced, bagasse surpluses are produced and sold to be used as fuel in other industries such as ceramics, orange juice plants, or as fodder in intensive calf breeding. In addition to that, a large amount of agricultural residues remain from sugarcane harvesting, approximately 12 tons of tops and leaves (dry basis) per hectare are available, practically useless which are mostly burned (a practice increasingly eradicated) or are left in the sugarcane fields. Besides the costs associated with its uses, sugarcane bagasse presents no cost in terms of production factors, and harvesting and transporting sugarcane straw from sugarcane fields has a low cost, initially estimated at around 1 US $/GJ (Hassuani et al. 2005).

Such large availability of lignocelullosic materials in the sugarcane industry, as well as in other agricultural and agroindustrial activities, is effectively primary primary energy resource to be better exploited. In this context and looking for new perspectives for bioenergy, there is a growing interest in using lignocelullosic materials for liquid fuel production. However, although these materials are accessible at reduced cost, the technology for their conversion in biofuels is still to be developed.

Currently, two technological routes are the most studied to crack the complex compounds of lignocelullosic biomass in more workable molecules to produce liquid fuels: hydrolysis and thermal gasification processes at low and high temperature, respectively. In this chapter we will present a review of gasification covering the basics aspects, the current state of the art, and some actual demonstration projects in operation or implementation. The objective is to summarize biomass gasification and synthesis processes for producing ethanol and other biofuels, as pointed out in Fig. 1, and set its perspectives of development.

2 Gasification of Biomass for Biofuels Production

The history of gasification, the partial combustion of solid fuels in controlled atmospheres to produce a low to medium heating value fuel gas, dates back to the seventeenth century, with the first attempts using coal. In 1788, Robert Gardner obtained the first patent related to gasification and during the nineteenth century many improvements were introduced into this technology, which became capable of fueling the first commercial models of stationary internal combustion engines

and, following the development of the auto industry, was also applied in light vehicles, trucks, and tractors. Considering these automotive applications, biomass gasification particularly, has been evolving since the 1930s, with the development of different equipment for charcoal and wood, mainly in Europe (Turare 1997). The interest in gasification dwindled after World War II but reborn with the oil shocks in the 1970s. Contemporary gasifiers range from small systems that supply gas for automotive internal combustion engines and small stationary units supplying combined heat and power (CHP) systems to larger scale gasifiers developed to generate power with gas turbines, at thermal power ratings of 10–100 MW and, more recently, to produce clean gas for the synthesis of liquid fuels (Bridgewater 1995). Gasification should not be confounded with pyrolysis, which is the thermal processing of biomass with only high temperatures, generally aiming to produce oils and heavy liquid fractions, sometimes called bio-oil.

In a broad definition, gasification is a thermochemical process for biomass conversion carried out at high temperatures, using reactors called gasifiers, in which solid or liquid organic substances are converted into gaseous products, usually called producer gas, synthesis gas, or syngas. The main syngas components are CO, H_2, CO_2 and water steam, along with the formation of light hydrocarbons and other volatile and condensable compounds as secondary products (Grabowski 2004). This process is appropriated for dry lignocelullosic materials, with a low content of inorganic components, generally discharged in the form of ashes. Gasification can be carried out by reaction of biomass with oxygen from the air or from steam, or even with pure oxygen, and using gasifiers at atmospheric pressure or pressurized. The heating of gasifiers can be direct, by partial oxidation of the biomass, or indirect, from external sources and using heat exchange mechanisms. According to the relative movement of the biomass under gasification, the gasifiers are basically designed as fixed bed (with gases flowing updraft or downdraft), fluidized bed (the biomass is kept in suspension by an upward flow of gasification gas), or moving bed, as explained in Tables 1 and 2 (Bridgewater 1995). In fact, there is a large range of gasifier types and the choice of the gasification technology will basically depend on the biomass to be processed, the type of product sought, and the size of the plant.

The feedstock composition also affects the synthesis gas composition, as indicated in Table 3, summing up of experimental results of gasification of charcoal and several agroindustrial by-products, obtained in a small scale downdraft fixed bed air-blown gasifier (Rajvanshi 1986). It is interesting to observe that when gasification is carried out with oxygen and steam, the heating value and combustible gases content increase, as could be expected.

The feedstock to be used in a gasifier usually requires a previous preparation, aiming basically to reduce its moisture and size. In the gasifier, after the initial phases of drying and partial volatilization of light compounds of biomass, the first group of reactions takes place at approximately 280–500°C, producing large quantities of tar and carbon dioxide, but with the increase of temperature, up to approximately 750°C, the tar components are cracked and some hydrogen is produced. Then, depending on the gasification technology, a partial oxidation of some

Table 1 Biomass gasifier types

Gasifier	Basic description
Fixed bed	
Downdraft or concurrent	Biomass moves down, gas moves down
Updraft or countercurrent	Biomass moves down, gas moves up
Cross-current	Biomass solid moves down, gas moves at right angles
Fluidized bed	
Single reactor	Low gas velocity, inert solid stays in reactor
Circulating	Inert solid is elutriated with product gas and recirculated
Entrained	Usually no inert solid; highest gas velocity of lean-phase systems
Twin reactor	Steam gasification and/or pyrolysis occurs in the first reactor; char is burned in the second reactor to heat the fluidizing medium for recirculation; either can be any type of fluidized bed
Moving bed	Mechanical transport of biomass; usually lower temperature processes
Other	
Rotary kilns	Good gas-biomass contact; careful design needed to avoid solids carryover
Cyclonic or vortex reactors	High particle velocities give high reaction rates

Source: adapted from Bridgewater (1995)

Table 2 Typical gas characteristics from different gasifiers

Gasifier type and operation	Gas composition (vol.%)					HHV (MJ/m^3)	Gas quality	Tars dust
	H_2	CO	CO_2	CH_4	N_2			
Fluidized bed, air-blown	9	14	20	7	50	5.4	Fair	Poor
Updraft, air-blown	11	24	9	3	53	5.5	Poor	Good
Downdraft, air-blown	17	21	13	1	48	5.1	Good	Fair
Downdraft, oxygen-blown	32	48	15	2	3	10.4	Good	Good
Multi-solid fluidized bed, oxygen blown	15	47	15	23	0	16.1	Fair	Poor
Twin fluidized bed gasification, oxygen and steam blown	31	48	0	21	0	17.4	Fair	Poor
Pyrolysis (for comparison)	40	20	18	21	1	13.3	Poor	Good

Source: adapted from Bridgewater (1995)

Table 3 Typical gas characteristics for different biomasses, downdraft air-blow gasifier

Product gasified	Composition (excluding N_2) (vol. %)				HHV (MJ/m^3)
	CO	H_2	CH_4	CO_2	
Charcoal	28–31	5–10	1–2	1–2	4.6–5.6
Wood (12–20% moisture content)	17–22	16–20	2–3	10–15	5.0–5.9
Wheat straw pellets	14–17	17–19	–	11–14	4.5
Coconut husks	16–20	17–19	–	10–15	5.8
Sugarcane bagasse	15–18	15–18	–	12–14	5.3
Corn cobs	19	16	6	–	6.3
Rice hulls pelleted	16	10	1	–	3.2
Cotton stalks cubed	16	12	3	–	4.3

Source: adapted from Rajvanshi (1986)

components can occur, releasing heat, to finally reach the last phase of gasification, at temperatures near 1,000°C, at which time some components are reduced, absorbing heat. In fact, the simultaneous reactions that occur in a gasifier are extremely complex and difficult to follow due to high temperature and short life span of some intermediate chemical products, but the efficiency of the process depends directly on how properly they are carried out. In some gasifiers the process is done at medium temperature and the biomass is just partially converted in gaseous products, while in high temperature gasifiers, a fully conversion of biomass is reached.

To give a simplified idea of the gasification process that follows the volatilization of the solid fuel, the following reactions summarize what occurs simultaneously (Rauch 2002):

$$C + \tfrac{1}{2} O_2 \rightarrow CO \quad (1)$$

$$C + H_2O \rightarrow H_2 + CO \quad (2)$$

$$C + O_2 \rightarrow CO_2 \quad (3)$$

$$CO + H_2O \rightarrow CO_2 + H_2 \quad (4)$$

$$C + CO_2 \rightarrow 2CO \quad (5)$$

$$C + 2H_2 \rightarrow CH_4 \quad (6)$$

From the above equations, it is possible to conclude that higher contents of steam in the gasification gas increase the tendency of hydrogen formation, while the increase of gasifier pressure facilitates the methane production (Nogueira and Lora 2004).

Considering cellulose gasification with oxygen in ideal conditions, in which the heat supplied from exothermic reactions is enough to promote gasification, the equivalence ratio, which expresses the amount of oxygen required for gasification relative to the amount required for combustion, is 0.244 and the gasification process can be exemplified as below, according Prins et al. (2007):

$$C_6(H_2O)_5 + 1.461O_2 \rightarrow 4.567CO + 1.295CO_2 + 0.138CH_4 + 3.958H_2 + 0.766H_2O \quad (7)$$

In regard to gasification efficiency, defined as the ratio between the total heating value of produced gas and the heating value of gasified feedstock, actual biomass gasifiers are less efficient than coal gasifiers, especially due to the high oxygen content of biomass feedstock, in which the atomic ratio O/C typically ranges from 0.5 to 0.8. Depending on the final temperature and operational condition, taking

into account the energy consumption in ancillary systems, the efficiency of biomass gasifiers using steam and oxygen as gasifying agents ranges from 66 to 74%, in the best cases (Prins et al. 2007).

By gasification, a heterogeneous material such as biomass can be transformed into a more uniform gaseous product suited to various applications. The straightforward application is as fuel in boilers and furnaces, but it can also be used in internal combustion engines, gas turbines, and biofuel synthesis which imposes the gas cleaning to meet the specifications required by each particular use. Cleaning can occur at low temperatures, for example by filtering (at approximately 200°C) and washing for removal of particulates and condensable materials after cooling. Cleaning may also be carried out at medium tohigh temperatures (350–400°C) for use in gas turbines and fuel cells, in this case usually using ceramic filters (Macedo et al. 2006).

Many of the technical problems encountered in the development of this technology were identified and partially resolved in the 1990s, including how to feed large quantities of comminuted biomass into pressurized reactors and the development of systems to clean the gas to meet gas turbine quality standards. Thus, biomass gasification can actually be used in gas turbines designed for gases with low calorific power. Nevertheless, for application in synthesis reactors to convert biomass gasification products into liquids fuels, the contaminant removal and the balanced composition requirements will certainly require further development in gasification technology and gas treatment.

The scale of production is a determinant factor of the economic feasibility of liquid fuels production using gasification technology, and a reason why the pressurized CFB gasification technology is preferred by some authors (Hamelinck et al. 2003; Larson et al. 2005). The gasification process should be such that the gas produced is rich in CO and H_2, the two main reactants in liquid fuel production. Thus, air injection should be avoided because it is not desirable for the gas produced to be diluted with nitrogen. Another particular concern in biomass gasification is the slag formation related to alkaline elements of ash, which imposes appropriated design of gasifiers and, in some cases, additives to control the slag viscosity (Coda et al. 2007).

An example of state of the art biomass gasification is the two-stage atmospheric pressure biomass gasifier developed by Batelle (Higman and Van der Burgt 2003), shown in Fig. 2.

In this gasifier, biomass initially reacts with steam and hot recycled sand in a fluidized bed to produce synthesis gas. Some unreacted char and sand exits the gasifier with the synthesis gas, and is captured in a cyclone separator, to be burned in a fluidized bed combustor where the sand is heated to high temperatures. Such sand is conveyed back to the gasifier to provide the heat necessary for converting the biomass into synthesis gas, which can reach a heating value near 17 MJ/N m^3. This gas can be cooled, cleaned, and compressed for use as fuel or synthesizing biofuels. One interesting application for this gasifier is in cogeneration schemes associated with conventional ethanol production from corn, allowing efficient use of the residues to cover energy demand and reducing the natural gas consumption (De Kam et al. 2009).

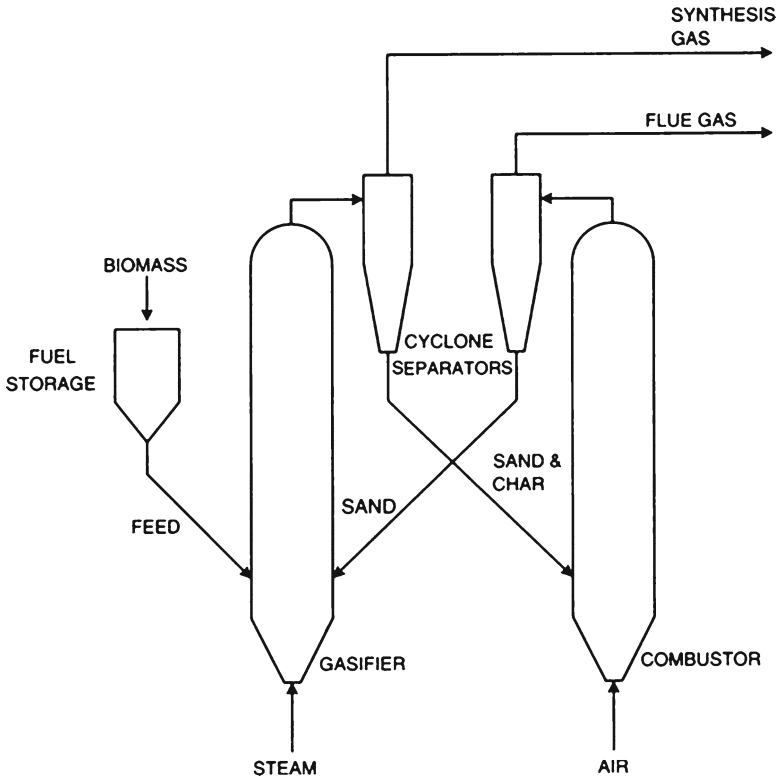

Fig. 2 Two-stage atmospheric fluidized bed gasifiers. *Source*: Higman and Van der Burgt (2003)

In addition to the technical aspects, a preliminary evaluation of biofuels production by gasification route points out that the gas production represents between 50% and 75% of overall cost, indicating the importance of gasifier optimization in order to reach the maximum gas yield at a low cost (Spath and Dayton 2003).

3 Synthesizing Biofuels from Syngas

Biofuels can benefit from the experience of the fossil fuel industry, where coal gasification has been in use for liquid fuel production for decades, but the high complexity of the processes involved in the biofuel synthesis, as mentioned, deserves attention. Despite the great effort involved in the development of this route, the synthesis phase is still an object of research and development, although for some research groups it seems to be one of the most feasible alternatives for biomass conversion in liquid fuels. Below, the synthesis process is presented considering the chemical and biochemical routes.

3.2 Chemical Synthesis

All significant discoveries in hydrocarbons synthesis were done in the beginning of the twentieth century. In 1902, Sabatier and Sanderens produced methane from CO hydrogenation by passing CO and H_2 over Ni, Fe, and Co catalysts. During that same period, hydrogen from syngas produced from steam methane reforming was commercialized. In a crucial development for explosives and fertilizers production, in 1910, Haber and Bosch discovered the synthesis of ammonia from H_2 and N_2 and the first industrial ammonia synthesis plant was commissioned in 1913. The production of liquid hydrocarbons and oxygenates from syngas conversion over iron catalysts was discovered in 1923 by Fischer and Tropsch (FT), whose process was in use in Germany between 1939 and 1945 to produce fuels from coal, generally called FT liquids. Currently, this technology has been applied in other plants in South Africa (by Sasol, since 1955), and more recently in Malaysia and Australia, using coal or heavy oil streams (Spath and Dayton 2003).

In the process of biofuels production, the synthesis gas coming from biomass gasification must pass through cleaning and reforming processes and, if necessary, adjustment of its composition, to be converted into fuel in a catalyzed reactor. Given that not all the gas is converted into fuel, the unconverted portion can be recirculated (to maximize fuel production), or it can simply be burned to generate electric power, in a Biomass Integrated Gas Turbine Combined Cycle (BIG/GT-CC) system, for example. The last option is known as once-through and it is considered the most economically viable approach when the electricity can be sold at convenient tariff (Hamelinck et al. 2005 and Larson et al. 2005). Figure 3 presents a general diagram of the production of different biofuels such as methanol, hydrogen, and Fischer-Tropsch liquids (FT diesel or FT gasoline), also indicating the possibilities for electricity production in steam and gas turbines. Other similar processes can be used to produce ethanol and dimethyl ether (DME).

Because gas produced by gasification may contain considerable quantities of methane and other light hydrocarbons, one option is converting these compounds into CO and H_2 at high temperatures and in the presence of a catalyst, generally nickel alloys. New catalysts, including the use of carbon-based nanoparticles, have been proposed with promising results (He and Zhang 2008). Another important factor is the H_2/CO ratio,

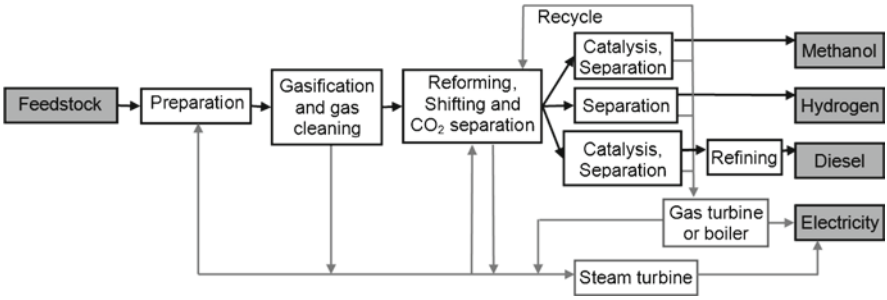

Fig. 3 Generic flowsheet for methanol, hydrogen, or FT diesel production, via gasification of biomass. *Source:* adapted from Hamelinck (2004)

which should be kept above 2 and adjusted for each type of biofuel, with less hydrogen in heavy fuels like diesel. This adjustment is done by the water–gas shift reaction, carried out in the presence of an iron-based catalyst (Van der Laan 1999):

$$CO + H_2O \rightarrow CO_2 + H_2 \tag{8}$$

The basic reactions involved in the production of each fuel are the following (Larson et al. 2005; Dermibas 2009):
For FT liquids:

$$nCO + (n + m/2)H_2 \leftrightarrow C_nH_m + n\,H_2O \tag{9}$$

For DME:

$$3CO + 3H_2 \leftrightarrow CH_3OCH_3 + CO_2 \tag{10}$$

For methanol:

$$CO + 2H_2 \leftrightarrow CH_3OH \tag{11}$$

As of today, especially in regard to ethanol production from synthesis gas, no commercial process exists, although research on this topic has been conducted during the past 90 years. Both homogeneous and heterogeneous catalytic processes can be used, but the first one is relatively more selective for ethanol. However, the need for expensive catalyst, high operating pressure, and the complex procedures involved for catalyst separation and recycling make these processes unattractive for commercial applications. Nevertheless, more recently, catalytic routes previously reported for the conversion of syngas to higher alcohols are in evaluation for ethanol production, with good perspectives, according to Subramani and Gangwal (2008).

There are three basic reactor designs for synthesizing biofuels: fixed bed, fluidized bed, and slurry bed. The first design provides low conversions with only a single passage and it is still difficult to extract heat. One example is the fixed bed tubular reactor known as the ARGE reactor, operating at 220–260°C and 20–30 bar. The second design offers greater conversions, but it involves a more complex operation, as in the high temperature circulating fluidized bed reactors, known as Synthol reactors, developed for light olefin production and operating at 350°C and 25 bar. Based on this concept, the Sasol Advanced Synthol reactor has been developed, as a fixed fluidized bed reactor with similar operating conditions as the Synthol reactor but at half the capital cost and size for the same capacity. The last kind of synthesis reactor, with a slurry bed, is the one that offers the highest conversion rates for processes with simple passage and lower methane production. One example is the low temperature slurry reactor with a solid catalyst suspended and dispersed in a high thermal capacity liquid, often a paraffin product. In this reactor, syngas is bubbled through the liquid phase allowing excellent contact with the catalyst while keeping the catalyst particles dispersed. Figure 4 presents schemes of these reactors, as used in actual FT plants (Spath and Dayton 2003).

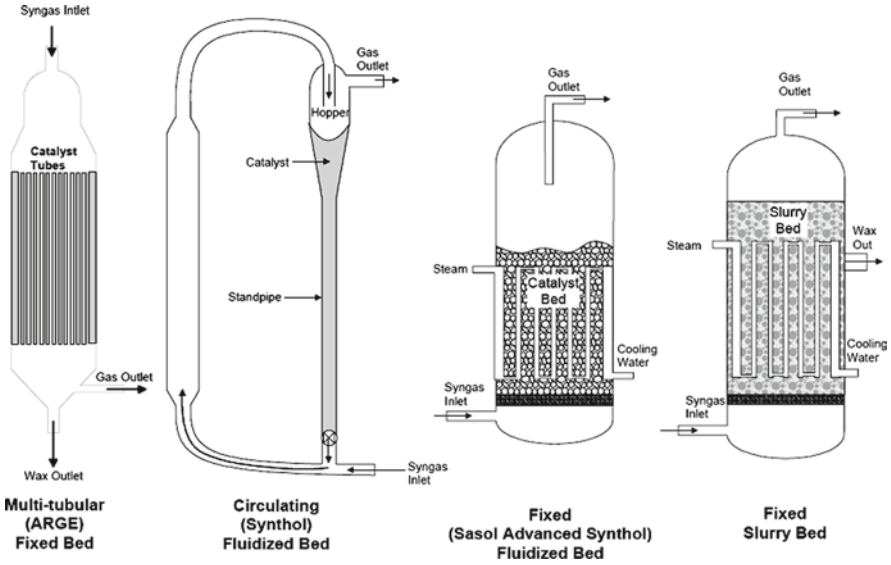

Fig. 4 Types of reactors for FT synthesis. *Source:* Spath and Dayton (2003)

Two basic features of FT synthesis are the simultaneous production of a wide range of hydrocarbon products (olefins, paraffins, and oxygenated products) and the intense release of heat from the highly exothermic synthesis reactions. Product distributions are influenced by reaction temperature, feed gas composition, pressure, catalyst type, and catalyst composition. Depending on the types and quantities of products desired, the most usual catalysts are iron or cobalt. Aiming at good performance, it is important to maintain the catalyst activity, which can be done by controlling the impurity levels in the synthesis gas. One of most studied catalyst poisons is sulfur, which is practically absent from biomass, however, other catalyst poisons include halides and nitrogen compounds (e.g., $NH_3 < 10$ ppmv, $NO_X < 0.2$ ppmv and HCN < 10 ppb), which mean a real problem with use of biomass synthesis gas (Spath and Dayton 2003). Even in the case of gasification with oxygen and steam, the nitrogen naturally present in the feedstock is a real concern in terms of synthesis gas contamination.

3.3 Biochemical Synthesis

Still under research and development, but with good perspectives if these efforts succeed, the biochemical synthesis associated to biomass gasification has been intensively studied in the last years (Klasson et al. 1992; Morrison 2004; Datar et al. 2004; Younesi et al. 2005, Rajagopalaan et al. 2002 among others). The basic idea is simple: while yeasts decompose sugar or starch to produce ethanol, some bacteria of genus *Clostridium*, such as *C. ljungdahlii* and *C. autoethanaogenum*,

can use H_2, CO, CO_2, and H_2O to synthesize acetate and ethanol in a fermentation process. The reactions involved in this process are (Datar et al. 2004):

$$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2 \tag{12}$$

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \tag{13}$$

$$6CO + 3H_2O \rightarrow CH_3CH_2OH + 4CO_2 \tag{14}$$

$$6H_2 + 2CO_2 \rightarrow CH_3CH_2OH + 3H_2O \tag{15}$$

The temperatures required for biosynthesis reactions range from 30 to 40°C at atmospheric pressure. The ethanol bioconversion rate is low because the interaction between the gas phase and bacteria in liquid phase is inherently limited. In the experimental studies, synthesis gas is cyclically bubbled into the solution containing the bacteria, and it may take more than 20 days to completely convert a unit volume of syngas intoto ethanol. The biosynthesis product is a liquid stream containing ethanol, acetate, water, and a small amount of other compound results, which should be submitted to through a series of distillation and dehydration cycles to obtain fuel grade ethanol. In a parametric study of biomass gasification/fermentation process, Piccolo and Bezzo (2009) conservatively assumed that 53.1% of CO and 18.8% of H_2 are converted in ethanol, reaching final mass concentrations in broth of 2.4% for ethanol and 0.4% acetic acid. With improvements, we believe that up to 90% of CO and 70% of H_2 can be converted into ethanol (Spath and Dayton 2003).

The current research efforts are mostly directed at enhancing microbial culture stability and improving ethanol-to-acetate ratios (Cotter et al. 2009). In addition to that, Piccolo and Bezzo (2009) point out the importance of evaluation whether a higher mass transfer may cause a higher concentration of inhibitors in the broth, as well as recommend improving the bacteria resistance to ethanol concentration in the broth in order to allow more concentrated solutions and decrease the energy requirement for ethanol recovery. In order to remove ethanol and reduce its inhibition effect on biosynthesis, new separation processes are under evaluation, using vacuum, gas stripping, membranes, and liquid extraction, also with potential interest for cellulose hydrolysis technologies (Cardona and Sanchez 2007).

4 Current Development of Ethanol Production by Biomass Gasification

Looking into the current state of this technology, significant development has been observed with the construction and operation of pilot plants, demonstration projects, and even some precommercial units. Based on the experience with biomass gasifiers

and in the oil synthesis industry, some analyses have been conducted in recent years to evaluate the possibilities and costs of these biofuels in the future, with stimulating prospects, although with a high level of uncertainty. In the case of FT liquids (gasoline and diesel), for example, if all technological problems were resolved, the overall efficiency could surpass 57%, considering the combined production of fuels (34% efficiency) and electricity (23% efficiency). Larson et al. (2009) estimated that biofuel produced by biomass gasification followed by FT synthesis would cost, in the medium term, approximately 15 US $/GJ. For the sake of comparison, the price paid to Brazilian producers of sugarcane ethanol varied between 3 and 9 US $/GJ in the period between 1990–2002 (Goldemberg et al. 2004).

Table 4 presents some values from the literature, including yields and costs of liquid biofuels produced by means of synthesis processes associated with biomass gasifiers (Seabra 2008; Piccolo and Bezzo 2009). Although the estimated ethanol cost in this table is beyond the current market value, indicating that more development is still necessary to reach full commercial feasibility, these values should be compared with care because they were estimated using different parameters and hypothesis with regard to financial conditions, feedstock costs, by-products value, and process performance, including the value of electricity produced in cogeneration schemes. For the particular case of sugarcane residual biomass in Brazil, recent analysis has shown that ethanol costs for thermochemical conversion (considering short-term performance parameters) would be competitive in the current scenario, essentially due to the low biomass cost (Seabra and Chum 2009).

Aiming to support the development of cellulosic ethanol technology, the U.S. Department of Energy applied US$385 million in grants in 2007 to cofinance six demonstration plants expected to produce 492 million liters of ethanol in 2012. Among them, three initiatives put forward will use gasification and one will use a mix of technologies, as indicated in Table 5. These plants are planned to begin operation circa 2011.

Table 4 Comparison of yields and costs for biofuel production from gasification route

Reference	Biofuel and synthesis process	Yield (liter/dry t)	Unitary capital cost	Feedstock cost	Biofuel cost
Piccolo and Bezzo (2009)	Ethanol biochemical	203	4.72 US$/liter/year	48 US $/t	1.12 US $/liter
Phillips et al. (2007)	Ethanol chemical	334	0.82 US_{2005}/liter/year	35 US $/US ton dry	0.27 US $/liter[a]
Larson et al. (2009)	FT liquids	170	606 US_{2003}/$kW_{input, LHV}$	3 US $/$GJ_{LHV}$	15.25 US $/$GJ_{LHV}$
	DME[b]	310 kg/dry t	667 US $\$_{2003}$/$kW_{input, LHV}$	3 US $/$GJ_{LHV}$	13.80 US $/$GJ_{LHV}$
Hamelinck and Faaij (2001)	Methanol chemical[c]	280–630	520–660 US $\$_{2001}$/$kW_{input, HHV}$	2 US $/$GJ_{HHV}$	8–12 US $/$GJ_{HHV}$

Source: adapted from Seabra (2008) and Piccolo and Bezzo (2009)

[a]Minimum ethanol selling price

[b]Considering reactor with recycle

[c]Range of values estimated for six different production concepts

Table 5 Demonstration plants of cellulosic ethanol using gasification route cofinanced by US Department of Energy

Company	Estimated investment	Project
Alico, Florida	US $33 million	To convert 770 tons/day of yard, wood, vegetative wastes and eventually sugarcane into synthesis gas and produce 52.6 million liters of ethanol a year and 6,255 kW of electric power, as well as 8.8 tons of hydrogen and 50 tons of ammonia per day
Range Fuels, Georgia	US $76 million	To process 1,200 tons/day of wood residues and wood based energy crops by gasification and fermentation to make annually about 150 million liters of ethanol and 34 million liters of methanol
Abengoa Bioenergy, Kansas	US $76 million	Using both biochemical and thermochemical routes, to process 700 tons/day of corn stover, wheat straw, milo stubble, switchgrass, and other feedstock to produce 43.5 million liters of ethanol annually and enough energy to power the facility, with the surplus energy supplied to the adjacent corn dry grind mill

Source: US Department of Energy (2007)

5 Final Comments

Although the gasification process is still in development for ethanol production, rivaling other technologies, some parameters point out that gasification is a consistent option for biofuels production and is actually able to play an important role in the future. In this direction, fundamental feasibility indicators such as life cycle energy ratios, relating (1) the energy available in biofuel with the fossil energy consumed (fossil energy ratio), and (2) the energy available in biofuel with the total primary energy consumed (primary energy ratio) are respectively estimated as 16 and 0.35 for ethanol production by gasification and chemical synthesis (Spath and Dayton 2003). These values were estimated based only on technical data, without inferences of prices and costs, representing sound indicators of long-term feasibility. The first ratio indicates a very positive leverage of solar energy and the second one, which is in the same range of many other conversion processes in evaluation, indicates a reasonable conversion efficiency, independent of any economic aspects.

As reviewed in this chapter, innovative bioenergy systems are certainly interesting and have a significant volume of applied technology; however, their economic feasibility has not been demonstrated and the knowledge of their performance and costs, as well as their optimization conditions for design and efficient operation of plants is still in development, in spite of the impressive work already completed. Preliminary economic analyses showed that assigning a value to their ability to mitigate climate change is important to promote their economic viability compared with conventional energy sources, but only if their technical feasibility on commercial scales is effectively confirmed.

The expectation is that biomass gasification could lead to the production of both liquid biofuels, mainly for automotive use, and bioelectricity on a large scale in the medium to long term. Thus, to maintain the research and development efforts in this field is crucial.

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Part II
Plant Cell Walls, Enzymes, and Metabolism

Chapter 4
Hemicelluloses as Recalcitrant Components for Saccharification in Wood

Takahisa Hayashi and Rumi Kaida

1 Introduction

Cellulose is a linear polymer consisting of more than 2,000 1,4-β-glucopyranosyl residues. The glucosyl residues form intramolecular hydrogen bonds at O3–O5′ and O6–O2′. Therefore, each glucosyl residue is bound to its neighbors by three bonds consisting of one covalent bond at C1β–C4′ and two hydrogen bonds at O3–O5′ and O6–O2′. Each glucosyl residue is oriented at an angle of 180° to the next residue of the chain, which might be synthesized from two residues at a time during cellulose biosynthesis. Since individual strands of cellulose are intrinsically less hydrophilic than other soluble polysaccharides, crystals tend to form with extensive intra- and intermolecular hydrogen bonds and complex, three-dimensional structures. In these crystals, each parallel glucan strand is situated between hydrophobic ribbon faces by both hydrophobic bonds and intermolecular hydrogen bonds (O6–O3′). Glucan forms a nanofiber, which associates to form bundles of compact lattices made up of hydrophobic and hydrogen bonds. In the natural crystals (cellulose I), the cellulose strands are parallel and form monoclinic cellulose I (I_β) (Hackney et al. 1994). There are also the noncrystal regions, which contain multiple intercalations of some paracrystal glucans. Cellulose microfibrils consist of many paracrystal 1,4-β-glucans, which form nanofibers 3–4 nm in width and thickness. The surface glucans of these nanofibers may be irregularly intercalated with hemicelluloses to various degrees so that each nanofiber includes anywhere from one to several glucan layers (O'Sullivan 1997).

There is no enzyme equivalent to cellulase which can hydrolyze natural lignocellulose completely in a short time. It has been suggested that it is hard for enzymes to attack the crystal regions of cellulose, and also that the presence of lignin and hemicellulose prevents enzymes from attacking the cellulose. Cellulase

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does not easily hydrolyze 1,4-β-glucans that are intercalated with hemicellulose, nor does hemicellulase efficiently attack hemicellulose that is intercalated tightly into microfibrils in the xylem, because plant cell elongation and expansion further tighten the intercalation between 1,4-β-glucans and hemicellulose during growth. Once hemicelluloses are tightly intercalated between the microfibrils and the glucans, neither cellulase nor hemicellulase can attack the resulting complex composed from cellulose and hemicellulose. Hemicellulose association can only be prevented through its constitutive degradation in the inner surface of walls during wall assembly.

This review discusses the reasons why hemicellulose is a cause of the recalcitrance of wall polysaccharides to saccharification as well as of the recalcitrance of cellulose microfibrils to hydrolysis. Our aim was to assess hemicellulose intercalation as a cause of this recalcitrance.

2 Crystal Regions and the Presence of Lignin

Cellulose occurs in crystal and noncrystal regions as well as in association with lignin deposition in the secondary wall. Cellulose microfibrils are known to be particularly recalcitrant to saccharification where they occur in the crystal configuration or in the presence of lignin (Chen and Dixon 2007). Our question is whether the hydrolysis of cellulose in wood could be affected by the presence of crystal regions and/or lignin in any of several tropical trees. *Acacia mangium* is a fast-growing dicotyledonous tree grown on plantations in Indonesia, Vietnam, China, Brazil, and South Africa (Orchard and Maslin 2005). *Paraserianthes falcataria*, another dicotyledonous tree, belongs to the subfamily *Mimosoideae* of *Leguminosae* (Binkley et al. 2003), and could become one of the most useful tropical tree species in terms of biomass in industrial forests (Shivery et al. 2004; Kurinobu et al. 2007; Siregar et al. 2007). *Elaeis guineensis* (Oil palm) is a perennial monocotyledon widely planted for its oil in Malaysia and Indonesia, both of which are major producers of palm oil; these trees are cut down for replantation at ~25-year intervals.

Crystallinity was high for the xylems of *A. mangium* and *P. falcataria* and low for that of *E. guineensis* (Fig. 1); this is probably because oil palm trunk contains higher quantities of parenchyma cells than the other species do (Lim and Khoo 1986; Husin 2000). Based on the chemical analysis of their xylems (Table 1), the cellulose content was highest in *A. mangium* and lowest in *E. guineensis* (Kaida et al. 2009); this is because oil palm trunk contains a relatively large concentration of primary wall. The lignin levels in *A. mangium* and *P. falcataria* are within the range typically found in hardwoods, 20–26%, while that of *P. falcataria* was higher. The amounts of hemicelluloses in *A. mangium* and *P. falcataria* were comparable to one another (about 20%).

Woody meals of each of these three species were subjected to saccharification with cellulose preparation for 48 h. By the end of this time, *A. mangium* had only

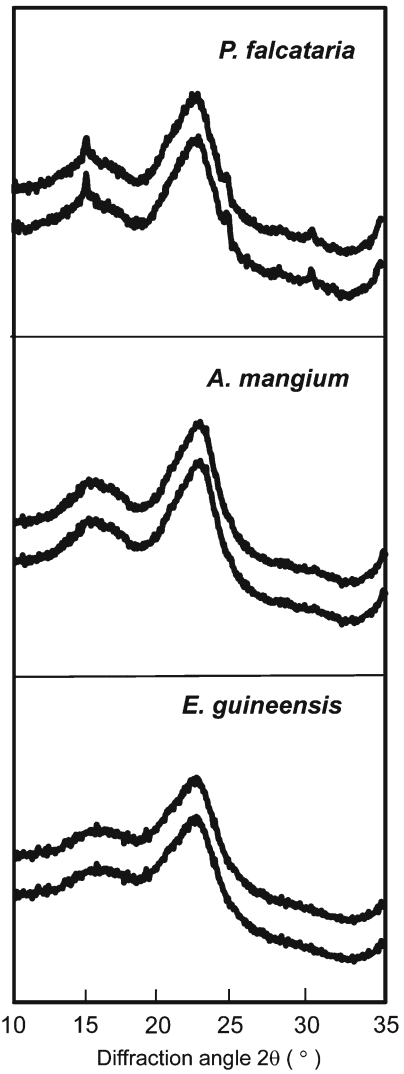

Fig. 1 X-ray diffraction profiles of xylems. Control xylem before enzymatic hydrolysis (*upper*) and the residual xylem (*lower*) after enzymatic hydrolysis for 48 h

released 8 mg of sugars, while *P. falcataria* had released 29 mg, and *E. guineensis* 18 mg (Table 1) (Kaida et al. 2009). X-ray diffraction analysis showed that enzymatic digestion of xylem preparations resulted in patterns of crystalline and noncrystalline regions similar to those seen in the undigested xylem preparations, due to two broad equatorial diffractions centered at ca. 15.5° and ca. 22° (Fig. 1). Thus the degradation of cellulose microfibrils occurred at equal rates in crystalline and noncrystalline regions at all loci.

When saccharification was accompanied by fermentation with yeast, ethanol production was higher in *P. falcataria* than in the other species, as was the degree of saccharification achieved. During this process, both saccharification and

Table 1 Components of wood from *Acacia mangium* and *Paraserianthes falcataria*, and of trunk from *Elaeis guineensis*, and their saccharification levels

	A. mangium	*P. falcataria*	*E. guineensis*
Components	mg/100 mg xylem		
Cellulose	59.7	52.5	45.9
Lignin	21.2	26.5	19.9
Hemicellulose[a]	20.1	21.0	34.4
Saccharification[b]	8.0	29.0	18.0

[a] Hemicellulose was determined as glucose by the phenol sulfuric acid method
[b] Saccharification was determined as reducing sugar by the Nelson–Somogyi method

fermentation occurred at high rates in *P. falcataria*, in spite of the fact that it has a higher lignin content than the other two species. This result shows that crystal regions and lignin do not always restrict the enzymatic saccharification of plant cell walls.

3 Xylan

Xylan fibers constitute a significant portion of the hemicellulose in the lignocellulosic components of wood in fast-growing trees (Baba et al. 2009), providing the strength of plant secondary walls (Bachner et al. 1993). Xylan is a complex polysaccharide, which consists of a backbone of xylose residues linked by β-1,4-xylosidic bonds. Significantly for our purposes, xylan is bound to lignin (Imamura et al. 1994; Lawoko et al. 2006), so that the constitutive degradation of xylan might also reduce lignin deposition in plant cell walls.

Xylanases are glycosyl hydrolases that catalyze a hydrolysis of the β-1,4-glycosidic bonds in xylan. The biotechnological application of xylanases has broadened recently, as xylan is an antinutritional material in monogastric animals, particularly poultry (Kimura et al. 2003). The stable expression of xylanase in a particular crop would be expected to improve the digestibility of that crop's cell wall fibers when the plants are used as forage. For this purpose, several transgenic plants have been generated expressing xylanases from microorganisms; not only can xylanases accumulate in the intercellular spaces of tobacco (Borisjuk et al. 1999; Komarnytsky et al. 2000), but they can also be targeted to chloroplasts or peroxisomes (Leelavathi et al. 2003; Hyunjong et al. 2006). These transgenic plants also have a potential application in our field, in the production of plants expressing recombinant xylanase to aid in cell wall saccharification, because xylan acts as a barrier to efficient cellulase function, and thus must be removed for efficient cell wall saccharification. When plant xylanase was overexpressed in poplar, xylan content was decreased to be less than half in the walls. However, no significant difference in lignin content was found between any of the transgenic lines and wild-type poplar.

Table 2 Levels of hydrolysis for wall polysaccharide and cellulose released during enzymatic hydrolysis of woods from poplars overexpressing xylanase, xyloglucanase, and cellulase

	Level of hydrolysis[a] (%)	
Poplar	Wall polysaccharide	Cellulose
Wild type	30	31
Transgenic overexpressing		
Xylanase	38	52
Xyloglucanase	48	57
Cellulase	37	46

[a]Level of hydrolysis was shown as the ratio of the amount of labile sugar to that of wall polysaccharide and the ratio of the amount of labile glucose to that of cellulose

Saccharification level in poplar woody meal was increased from 30% in the wild type to 38% in the transgenic poplar overexpressing xylanase (Table 2). Cellulose was also highly hydrolyzed in the same transgenic poplar, at up to 52% of the total cellulose content. Furthermore, xylan was almost completely hydrolyzed after 48 h. This is in agreement with the finding by O'Dwyer et al. (2008) that xylan hydrolysis was independent of cellulose digestion during the initial hydrolysis stage of poplar wood samples. When saccharification was accompanied by fermentation with yeast, ethanol production from the enzymatic hydrolysate was higher in the xylem overexpressing xylanase than in the wild type. Thus the constitutive degradation of xylan was able to accelerate the saccharification of poplar wood.

4 Xyloglucan

Xyloglucan consists of 1,6-α-xylosyl residues along a 1,4-β-glucan backbone. Species-specific differences occur in the distribution of additional branching galactosyl and fucosyl-galactosyl residues. Since the 1,4-β-glucan backbone can bind specifically to cellulose microfibrils by hydrogen bonds (Hayashi et al. 1994), xyloglucan probably contributes to the rigidity of the cell wall by cross-linking adjacent microfibrils. In fact, microfibrils seem to be coated with xyloglucan, which is located both on and between microfibrils throughout cell elongation.

The overexpression of xyloglucanase in poplar resulted in the cleavage of xyloglucans cross-linked with cellulose microfibrils and in the acceleration of stem elongation through a loosening of the wall (Park et al. 2004). The overexpression of this enzyme also causes wall density and cellulose content to increase (Park et al. 2004). Since cellulose deposition in the stem is enhanced in the secondary xylem of the transgenic lines as well as in the primary wall, the enhancement of cellulose deposition in transgenic poplar trees could perhaps be ascribed to their altered pattern of xyloglucan cross-links. It seems that cellulose formation is restricted by entanglement with xyloglucan and enhanced by the relaxation that results from

cross-linking xyloglucans. Nevertheless, no significant difference in lignin content was found between any of the transgenic lines and wild-type poplar.

Saccharification level in poplar woody meal was increased from 31% in the wild type to 48% in the transgenic poplar overexpressing xyloglucanase (Table 2). Cellulose was also highly hydrolyzed in the same transgenic poplar, at up to 57% of the total cellulose content.

The increased cellulase activity in the walls did not affect the level of cellulose, but rather loosened xyloglucan intercalation, which led to an irreversible wall modification (Park et al. 2003; Shani et al. 2004). Reducing xyloglucan content through the overexpression of cellulase could enhance not only growth rate but also enzymatic hydrolysis. Saccharification level in poplar woody meal was increased from 31% in the wild type to 37% in the transgenic poplar overexpressing *Arabidopsis* cellulase (Table 2). Cellulose was also highly hydrolyzed in the same transgenic poplar, at up to 46% of the total cellulose content. These results show that xyloglucan intercalated into cellulose microfibrils could be one of the major causes of lignocellulose's recalcitrance to saccharification.

5 Glucomannan

Glucomannan possesses a straight β-(1→4)-linked glycan composed of glucose and mannose in various ratios. Species-specific differences may occur in the ratio of glucose residues to mannose residues. Since the glycan may bind specifically to cellulose microfibrils by hydrogen bonds, the function of the polysaccharide is unknown (Schroder et al. 2009). Cellulose microfibrils seem to be coated with glucomannan as well as xyloglucan, which is located both on and between microfibrils.

Plant cellulase can hydrolyze paracrystalline sites of cellulose microfibrils, leading to the release of glucomannan and xyloglucan (Shani et al. 2004; Park et al. 2003). Overexpression of *Arabidopsis* cellulase decreased the amount of glucomannan as well as that of xyloglucan in poplar. A decrease in glucomannan due to the overexpression of cellulase could enhance not only growth but also enzymatic hydrolysis. The saccharification level in poplar woody meal was increased from 31% in wild-type poplar to 46% in the transgenic poplar overexpressing *Arabidopsis* cellulase (Table 2). It should be noted that the increase in saccharification that is caused by the overexpression of cellulase is due not only to a decrease in glucomannan content but also to a decrease in xyloglucan content.

6 Conclusion

In this chapter, we have shown that enzymatic saccharification is accelerated after the constitutive degradation of specific hemicellulases in xylem. Overexpression of xyloglucanase resulted in the apparent acceleration of hydrolysis of wall polysaccharides and cellulose.

The constitutive removal of hemicellulose may increase the effectiveness of cellulase's attack by two distinct mechanisms: first, by increasing the pore space in the walls, and second, by increasing the incidence of paracrystalline sites of microfibrils where xylan and xyloglucan cannot be intercalated. Increasing the pore space creates more physical space, which allows the enzyme to access cellulose more easily, while increasing the incidence of paracrystalline sites favors the binding of cellulase to microfibrils. These modifications to the wall structure could facilitate saccharification. Although it is impossible to remove xyloglucan once it has been intercalated into cellulose microfibrils, its intercalation can be prevented, but only during the synthesis of microfibrils when cell walls are growing.

Plant biomass of a particular species can be made more easily degradable into monosaccharides by enhancing certain relevant qualities through genetic engineering. Now that the structure and function of wall components are beginning to be understood, it is becoming possible to apply *in fibril* and *in wall* modifications (Hayashi et al. 2010) to generate cell walls that are strong enough to support the plant during growth yet also easy to digest with an enzyme preparation for saccharification after harvest.

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Chapter 5
Topochemistry, Porosity and Chemical Composition Affecting Enzymatic Hydrolysis of Lignocellulosic Materials

Adriane M.F. Milagres, Walter Carvalho, and Andre Ferraz

1 Introduction

Lignocellulosic materials such as sugarcane bagasse represent a lowcost source of carbon for biofuel and chemical production, including cellulosic ethanol. Despite its low cost and availability, bagasse presents several technical challenges for its conversion to monomeric sugars suitable for fermentation processes. It is highly recalcitrant, which requires efficient pretreatment for enzymatic hydrolysis. Both pretreatment and enzymatic hydrolysis have frequently been highlighted as the most costly steps in the bioprocessing of this lignocellulosic material.

Several technologies for the pretreatment and disruption of the lignocellulose matrix have been developed worldwide. Acid prehydrolysis and some analogous technologies being reviewed in this chapter have been to date preferred. Such pretreatments aim essentially to remove hemicellulose from the lignocellulose template, providing a less structured material with increased porosity, which is highly desirable for the subsequent enzymatic hydrolysis of the remaining cellulose. Unfortunately, the presence of lignin in plant cell walls or even in acid pretreated lignocellulose hinders the efficient enzymatic breakdown of cellulose. In this regard, research groups worldwide are involved in multidisciplinary projects aiming to select or to create lignified plants with reduced lignin contents. In these cases, the idea is to mitigate the need for severe pretreatment because they are costly and often quite harsh, resulting in degradation and loss of substantial parts of the lignocellulose substrate.

Putting information together from several research areas, it is easy to realize that pretreatments and genetic engineering programs focused on plant improvement for cellulosic ethanol production aim to enhance the cell wall porosity in lignocellulosic materials. The challenge for this development is to define how much lignin and/or hemicellulose removal would be necessary for efficient enzymatic hydrolysis of

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cell wall components. Breakthrough advances in this area must be accompanied by ultrastructural and molecular investigations of the lignocellulosic material. In this context, the current chapter compiles information on the topochemistry, porosity, and chemical composition determining successful enzymatic hydrolysis of lignocellulosic materials.

2 Cell Wall in Lignified Plants: Structure, Chemical Composition, and Recalcitrance to Natural Decay

Lignocellulosic materials represent the most significant fraction of the terrestrial phytobiomass, the largest source of organic compounds in the biosphere. They are composed by three major fractions, namely cellulose, hemicelluloses, and lignin. Together, these fractions usually make up more than 90% of the material dry mass (Pandey et al. 2000).

Cellulose, the most abundant constituent of the plant cell wall, is a homopolysaccharide composed by D-glucose units joined together by β(1→4) glucosidic linkages. It may present a degree of polymerization higher than 10,000. Its linear structure, conferred by the configuration of the glucosidic bonds, facilitates the formation of intra- and intermolecular hydrogen bonds and leads to the aggregation of the cellulose chains in "elementary fibrils" with a high crystallinity degree. These aggregates are highly resistant to tension, make cellulose insoluble in a large number of solvents, and explain, at least in part, its resistance to microbial degradation (Ding and Himmel 2006; Matthews et al. 2006).

In turn, hemicellulose is a name given to heteropolysaccharides composed by D-glucose, D-galactose, D-mannose, D-xylose, L-arabinose, D-glucuronic acid, and 4-O-methyl-D-glucuronic acid units. These heteropolysaccharides are branched, may be acetylated, and usually present a degree of polymerization below 200. In soft woods, galactoglucomannans and arabinoglucuronoxylans are the main constituents. In hard woods, 4-O-methyl-glucuronoxylans and glucomannans are commonly found. Grasses, such as sugarcane, present 4-O-methyl-glucuronoarabinoxylans as the main hemicellulosic polysaccharide (Carpita 1996; Kuhad et al. 1997; Willfor et al. 2005a, b).

Lignin, on the other hand, is a complex aromatic macromolecule formed by radical polymerization of phenyl-propane alcohols (*p*-coumarilic, coniferilic, and synapilic). It is usually classified as softwood lignin when the coniferilic alcohol derivatives prevail, hardwood lignin when both coniferilic and synapilic alcohol derivatives coexist, and grass lignin when it contains important amounts of *p*-coumarilic alcohol derivatives. The most abundant nonpolysaccharidic component of the lignocellulose can form covalent bonds with the constituents of the hemicellulose, most of them esterbonds among lignin constituents and side chain arabinosyl residues. Lignin and hemicelluloses involve the cellulose microfibrils, conferring protection against chemical and/or biological degradation. While the cell walls of grasses present the lowest contents of lignin, those of softwoods are the richest in this

component (Kuhad et al. 1997). The mechanism that explains the protective effect of lignin against polysaccharide hydrolysis remains uncertain although a number of factors such as the degree and type of cross-linkage to polysaccharide, the diversity of structures found in the lignin component, and the distribution of phenolic polymers through the cell wall are important.

It is important to differentiate the components of the plant cell wall, polysaccharides and lignin, from those compounds extraneous to the fiber. Many of the latter components are readily soluble in water and/or neutral organic solvents and, therefore, are collectively named extractives. Others, such as proteins and some salts on the other hand, can be completely insoluble in the solvents used for the removal of the extractives. Commonly associated with properties such as color, smell, and taste, the extractives are composed by low molar mass compounds (sugars, aromatics, waxes, fatty acids, and resins) involved in the metabolism of the plant cell (Browning 1967).

From a technological point of view, the monosaccharides contained in the cellulose (glucose) and in the hemicellulose (xylose, arabinose, glucose, mannose, and galactose) represent the substrates that can be used for the production of ethanol and other goods by fermentative means. However, as shown in Fig. 1, the close association among the three main fractions (cellulose, hemicelluloses, and lignin) is such that it imposes great difficulties to recover the constituent sugars in the form of monomers with high purity (Sun and Cheng 2002). As shown in Fig. 2, the wall of

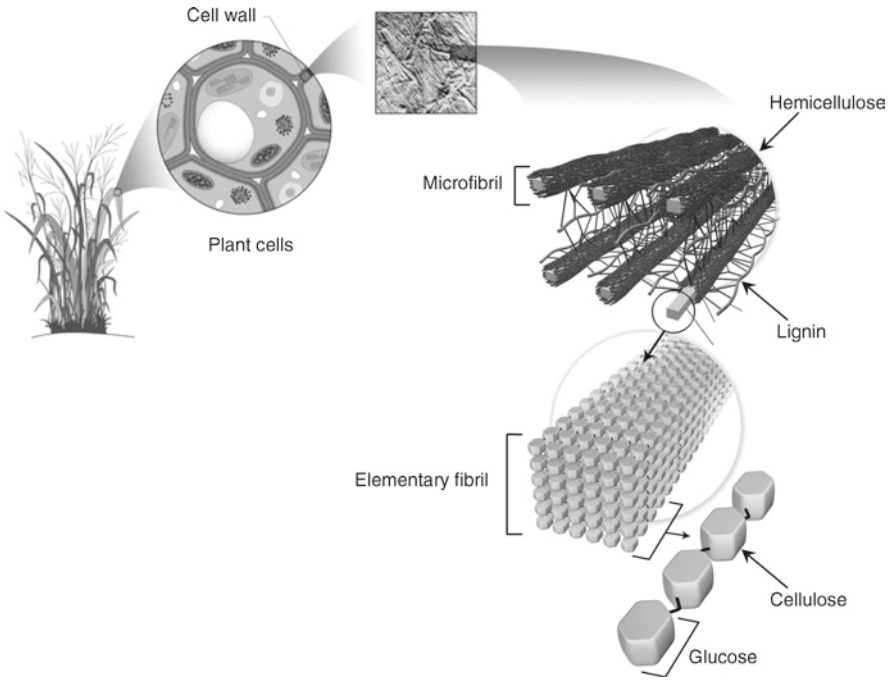

Fig. 1 Architecture of the plant cell wall (Adapted from http://genomics.energy.gov)

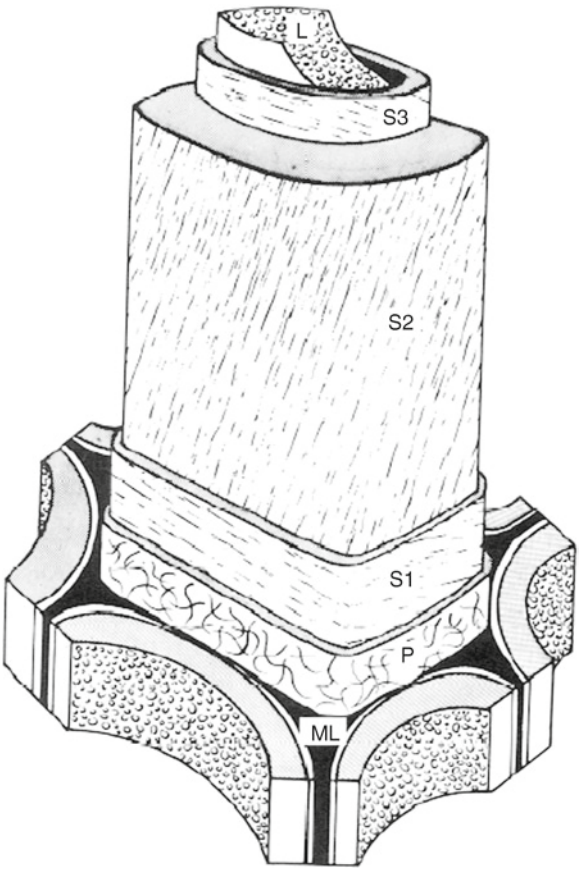

Fig. 2 Schematic representation of a generic plant cell: middle lamella (ML), primary wall (P), outer secondary wall (S1), middle secondary wall (S2), inner secondary wall (S3), lumen (L)

a lignified cell is composed by several layers, namely primary wall (P), outer secondary wall (S1), middle secondary wall (S2), and inner secondary wall (S3). These layers are formed by cellulosic microfibrils arranged in the space in particular orientations characteristic for each layer. Hemicellulose and lignin work as an amorphous matrix involving these elementary microfibrils (Fengel and Wegener 1989).

At this point, it is worth mentioning that no definitive model of the cell wall exists, particularly one that relates the cell wall composition to its porosity. For example, past studies by Carpita et al. (1979) have shown that even cells with only the nonlignified primary walls presented very limited pore diameters (in the range of 35–52 Å). When the authors compared their findings with other studies carried out with lignified cell walls, they were surprised to observe that cells containing only the thin (about 0.1 μm) primary wall presented pore diameters similar to the ones observed in lignified cells with thick secondary walls.

Owing to the recalcitrance of the cell walls from lignified plants, only a small group of microorganisms are able to degrade these materials in nature. They comprise mainly soft-, brown-, and white-rot fungi which use an intricate

extracellular system to decompose the wood cell wall macromolecules into small compounds that can pass across the cell membrane and be used in the metabolismtries (Kirk and Cullen 1998). This extracellular system is based on oxidative and hydrolytic enzymes such as lignin peroxidase (LiP) (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13), and "versatile" peroxidase (EC 1.11.1.7) that oxidize Mn(II) but also oxidize LiP substrates (Hammel and Cullen 2008), as well as cellulases and hemicellulases discussed in detail later in this chapter. Nonetheless, most of these enzymes are too large to penetrate intact lignified cell walls (Flournoy et al. 1991), thus requiring, for example, some type of cell wall cutdown to operate inside S2 layers. In fact, fungi use cooperative mechanisms, which first involve low molecular mass compounds, such as metal chelators (Goodell et al. 1997) or enzyme mediators (Hammel and Cullen 2008) capable to precede enzymes into the microvoids of lignocellulose structure and opening up the cell wall. These intricate metabolic routes, in wood decay fungi, could be seen as a "natural" pretreatment step developed to overcome the recalcitrance of lignified wood cell walls.

3 Topochemistry, Porosity, and Chemical Composition Determining Successful Enzymatic Hydrolysis of Lignocellulosic Materials

Some substrate characteristics are critical for limiting cellulose and hemicellulose hydrolysis. Limited porosity of lignified cell walls hinders enzyme infiltration in a nonpretreated lignocellulosic material (Grethlein 1985). The amount of crystalline cellulose in the material is also relevant, because the higher its amount the lower is the enzymatic hydrolysis rate (Jeoh et al. 2008). Topochemical distribution of lignin and hemicelluloses in cells is noteworthy because these components encapsulate cellulose microfibrils hindering cellulase domains to adsorb on cellulose for initiating enzymatic hydrolysis (Yang and Wyman 2004; Kristensen et al. 2008). Additional recalcitrance of lignocellulosic materials to enzymes is related to the variety in the thickness of the cell walls in different tissues, to the presence of epidermal tissue in some plant stems and to the diversity in the arrangement and density of vascular and fiber bundles (Mooney et al. 1999; Himmel et al. 2007).

The increase in the cellulase reactivity toward cellulose attributed to pretreatment of lignocellulose has usually been related to the creation of surface openings or internal slits, voids, or spaces by the removal of other cell wall components, enhancing the direct physical contact between the enzymes and the substrate (Himmel et al. 2007). For example, an acid pretreatment that removes hemicellulose to allow more cellulase-cellulose interaction, coupled with a way to physically disrupt the crystallinity of the cellulose structure, would greatly improve the enzyme digestibility of the biomass sample. In accordance, Bertran and Dale (1985) showed that the lower the initial crystallinity of cellulose, the higher the extent of conversion to soluble sugars. Recent research evaluating the selective lignin removal from wood cell walls

by ionic liquids clearly demonstrated a direct correlation among the levels of lignin removal from the cell walls and the increased digestibility of the remaining material by cellulases (Fig. 3). The same work provided evidence of improved digestibility of the substrate as a function of reduced contents of crystalline cellulose (Lee et al.

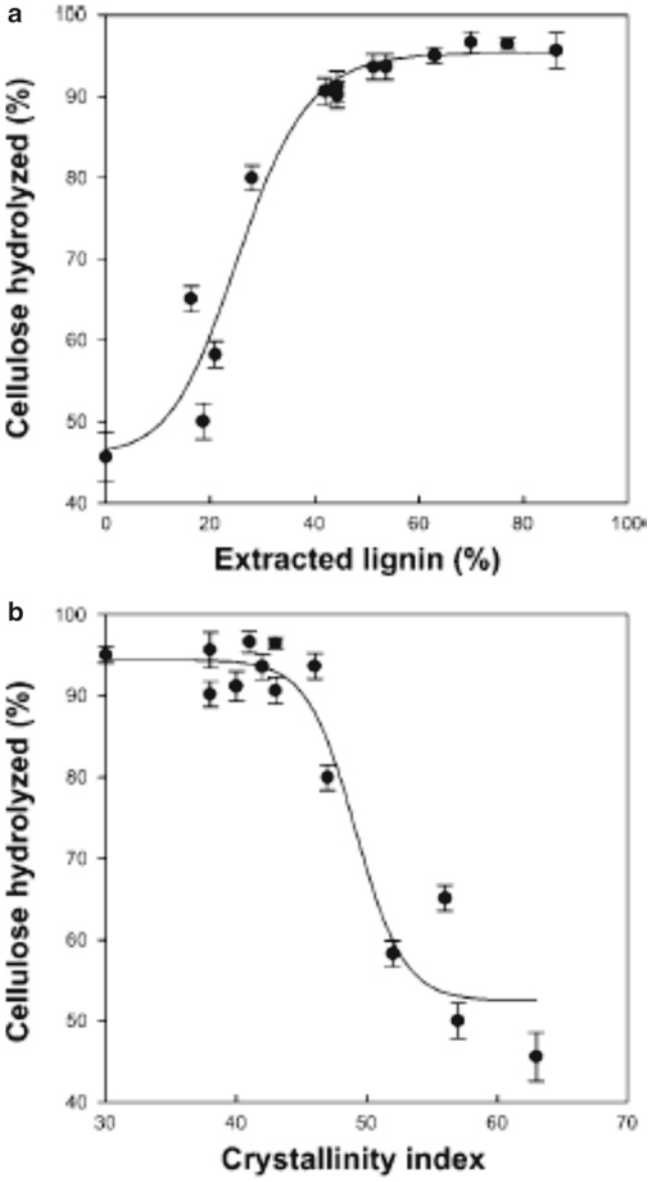

Fig. 3 Relationship between lignin content (**a**) and cellulose crystallinity (**b**) of pretreated wood flour and its digestibility (Reproduced from Lee et al. 2009)

2009). These data are in close agreement with past research published by Ramos et al. (1992). In this case when 80% of the lignin was removed the cellulose hydrolysis was largely improved. Furthermore, due to the lower lignin content, a sixfold lower enzyme loading was required to obtain the same degree of cellulose conversion. According to Silverstein et al. (2007), alkaline pretreatment of lignocellulosic materials can increase the cell wall porosity through delignification, breaking down some ester bonds cross-linking lignin and xylan, and causing swelling of microfibrils.

The characterization of the cell wall pores (i.e., size and size distribution) has been proposed as a means to predict the reactivity of the substrate to enzymatic hydrolysis (Mooney et al. 1998; Sanjuán et al. 2001; Jeoh et al. 2008). Past seminal work performed by Grethlein (1985) showed that dilute acid pretreatment of several lignocellulosic materials provided increased porosity in the substrate. In some cases, he observed a large increase in the volume of pores which were accessible to a solute with the size of a cellulase (diameter of 51 Å). Walker and Wilson (1991) also reported that during the process of cellulose fragmentation, there was a threefold increase in the specific surface area of the substrate. More recently, Ishizawa et al. (2007) corroborated that dilute acid pretreatment of lignocellulosic materials increased the volume of pores accessible to cellulases in corn stover. The same authors presented evidence that porosity may distinguish lignocellulosic substrates of low digestibility from those of high digestibility; however the results also indicated that porosity may not be the unique variable affecting the overall yields of ethanol production from lignocellulosic materials.

Another topic requiring attention is the conservation of the structure of the cell wall through pretreatment not being in accordance with the general perception that pretreatments must disrupt the structure of the cell wall in order to increase its accessibility to enzymes. For example, only partial hemicellulose removal and lignin relocalization are sufficient factors for increasing the digestibility of hydrothermally pretreated wheat straw (Palonen et al. 2004). These authors claimed that these factors were more important than the rupture of the skeletal cell wall structure and modification of cellulose crystallinity. Their results showed that it is possible to sufficiently pretreat wheat straw without disrupting the cell wall. Furthermore, they pointed out that only modest pretreatment suffices to promote an efficient enzymatic digestion of the polysaccharides, provided that mixing is efficient. Another example can be obtained from related studies on wood biodegradation by white- and brown-rot fungi. Machuca and Ferraz (2001) demonstrated that these fungi produce several extracellular enzymes when wood chips underwent solid state fermentation. The profiles of enzymatic activities produced during wood decay varied among the different fungi studied, but some general features were noted. All these fungi produced polysaccharide hydrolytic activities, but brown-rot fungi produced higher levels of cellulases and xylanases than did white-rot fungi. On the other hand, phenoloxidases were only found in white-rot fungal extracts. In general, high enzymatic activity did not necessarily result in large amounts of enzyme-specific wood component removal. Nevertheless, the extent of polysaccharide degradation was closely dependent of the extent of lignin removal.

4 Lignocellulose Pretreatment by Dilute Acid Hydrolysis and Analogous Technologies

Several strategies for the conversion of lignocellulosic polysaccharides into fermentable sugars have been demonstrated in bench and pilot scales. The general concept involves the pretreatment of the raw material followed by its enzymatic hydrolysis (Jorgensen et al. 2007).

Although pretreatment can be performed following a number of different principles, its goals are to remove or alter the hemicellulose and/or the lignin, to increase the pore volume and the internal surface area, and to decrease the degree of polymerization and crystallinity of the cellulose. Such alterations increase the enzymatic digestibility of the pretreated material and, hence, improve the yield on fermentable sugars (Zhang and Lynd 2004; Wyman et al. 2005; Mosier et al. 2005).

Ideally, the pretreatment should: (1) maximize the enzymatic digestibility of the pretreated material; (2) minimize the loss of sugars; (3) optimize the production of by-products (lignin, for example); (4) minimize capital and operating costs and; (5) be effective on multiple feedstocks (Holtzapple and Humphrey 1984).

As the plant cell wall is naturally impermeable to large molecules, including proteins such as cellulases, it is believed that for the efficient enzymatic saccharification of the cellulose, reagents that include (but are not restricted to) acids and bases have to be used to solubilize the hemicellulose and/or the lignin and, thus, increase the porosity of the matrix (Gould 1984; Schell et al. 1991). Purely physical pretreatments such as milling can also be used to increase the reactivity of the pretreated material toward the hydrolytic enzymes. Although usually very unfavorable in terms of energy consumption, these treatments can increase the available surface area and decrease the cellulose crystallinity and, thus, favor the enzymatic saccharification (Emmert and Rivers 1987). Biological pretreatments with fungi and bacteria that promote selective degradation of the lignin and/or the hemicellulose represent another option, but the biodegradation rates are usually very slow (Eriksson et al. 1980). Therefore, pretreatments that combine physical and chemical principles usually represent the best options to fractionate the lignocellulose (Ramos 2003).

Among the different methods of pretreatment, hydrolysis of the hemicellulose with dilute acids at high temperatures, followed or not by sudden decompression (explosion), is one of the leading technologies (Wyman et al. 2005). It represents a pretreatment strategy widely reported in the literature, successfully tested in pilot scale and with potential for application in commercial scale (Jorgensen et al. 2007).

During the dilute acid hydrolysis, the lignocellulosic material is mixed with an acid aqueous solution (H_2SO_4, typically), heated to the desired temperature and, after a certain residence time, cooled to room temperature. As illustrated in Fig. 4, most of the hemicellulose is removed from the microfibril (by acid-catalyzed hydrolysis of the heteropolysaccharides). Acetyl groups linked to the heteropolysaccharides are also cleaved and, therefore, act as hydrolysis cocatalysts. Lignin, on the other hand, is only removed to a limited extent from the material during the pretreatment, but rather redistributed on the fiber surfaces due to softening and

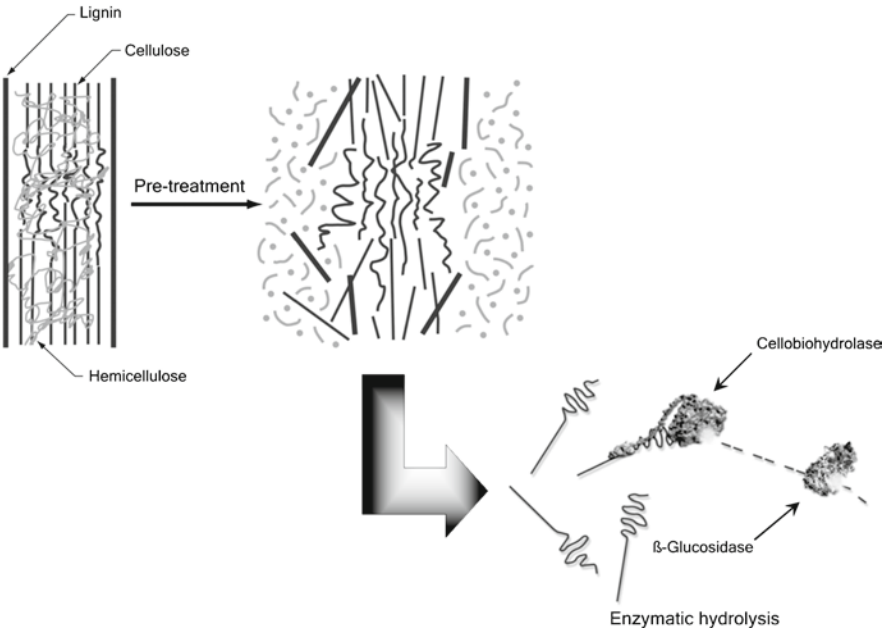

Fig. 4 Structural alterations in the architecture of the cellulosic microfibril caused by the pretreatment with dilute acid, followed by the enzymatic hydrolysis of the cellulose (Adapted from http://genomics.energy.gov)

depolymerization-repolymerization reactions. The removal of the hemicellulose and the redistribution of the lignin increase the pore volume and the internal surface area of the pretreated material, facilitating the accessibility of the enzymes for the subsequent cellulose hydrolysis (Schell et al. 2004; Lloyd and Wyman 2005; Kabel et al. 2007; Himmel et al. 2007; Li et al. 2007).

Depending on the type and conditions used during the pretreatment, hemicellulosic polysaccharides may remain in the pretreated material and hinder the enzymatic hydrolysis of the cellulose. In such situations, in addition to cellulases, hemicellulases are required for an efficient digestion of the pretreated material (Jorgensen et al. 2007).

5 Lignin-Depleted Plants for Improved Enzymatic Hydrolysis

With the advance of molecular biology and genetic engineering of plant species, lignin-depleted plants become a reality. This advent is critical for the further development of biotechnological processes aiming cellulosic ethanol production. For example, one study with fibers from transgenic alfalfa diminished in lignin content yielded nearly twice as much sugar based on direct enzymatic hydrolysis. The authors down regulated the lignin biosynthetic pathway at six different steps and compared the saccharification efficiencies of the six classes of transgenics. Suppression of genes

led the plants to produce less than half of the lignin found in the wild-type culture, which favored the hydrolysis of the polysaccharides at mild conditions (Chen and Dixon 2007). In wild-type alfalfa, plant maturation decreases cell wall digestibility owing to the accumulation of highly lignified xylary tissue (Grabber et al. 2002). In this study, the authors showed that xylem walls contained 28% lignin, compared with 15% lignin in nonxylem walls. Fungal enzymes hydrolyzed 22% and 73% of the structural carbohydrates in xylem and nonxylem walls, respectively, demonstrating unequivocally that lignification decreases cell wall digestibility.

More recently, Grabber et al. (2009) demonstrated that artificially lignified cell walls from maize had their digestibility to ruminal biota decreased as a function of increased lignification. Based on the use of different lignin precursors, the authors also concluded that the selection or engineering of plants for reduced lignification or ferulate-lignin cross linking will improve fiber fermentability more than shifting lignin composition, for example, by selecting high syringyl contents in lignified plants. The same group has shown that incorporation of coniferyl ferulate into lignin provides a new type of lignified cell wall (prepared in vitro from maize cells suspension) containing ester linkages connecting lignin side chains (Fig. 5). That alteration enhanced enzymatic degradation of the cell walls as well as lignin removal by mild alkaline treatment (Grabber et al. 2008). An immediate proposal derived from those studies is to use diverse esterases in saccharification processes not only to provide hemicellulose debranching, but also to help with delignification of these type of engineered plants. If this kind of lignin becomes available in engineered plants, it will be easy to advance that simple alkaline pretreatment which would yield a delignified pretreated substrate susceptible to efficient hydrolysis by a polysaccharidases cocktail.

6 Enzymatic Hydrolysis of Lignocellulosic Materials

Cellulose can be hydrolyzed into glucose residues either by enzymatic or acidic treatments. There are some limitations associated with the hydrolysis procedures that need to be overcome. In the case of acid hydrolysis, severe reaction conditions

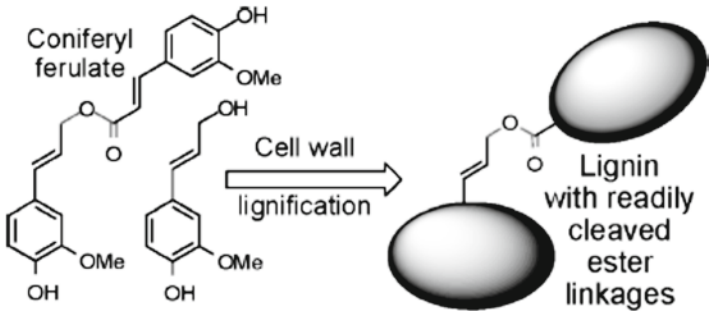

Fig. 5 The use of coniferyl ferulate as monolignols can yield lignin with ester-interunit linkages (Reproduced from Grabber et al. 2008)

are usually necessary (high temperature and acid concentration). This means that part of the sugars released from the polymer is degraded before the end of the reaction. In addition, the chemical hydrolysis usually produces small amounts of side products that can inhibit the subsequent fermentation step (Oliva et al. 2006). Enzymatic hydrolysis is an ideal approach for converting lignocellulose into sugars because mild reaction conditions can be used resulting in negligible by-products at high sugar yields. However, enzymatic hydrolysis depends on optimized conditions for maximal efficiency (hydrolysis temperature, time, pH, enzyme loading, and substrate concentration) and suffers from end product inhibition and biomass structural restraints (Ramos 2003).

The enzymatic hydrolysis of cellulose requires at least three classes of cellulolytic enzymes: endo-β-1,4-glucanases (E.C. 3.2.1.4), cellobiohydrolases (E.C. 3.2.1.91), and β-glucosidases (E.C. 3.2.1.21) belonging to different families (Bayer et al. 2004). High cellulase loadings are required to achieve reasonable rates and yields. Thus, finding paths to reduce cellulase loadings would be particularly effective in lowering the process costs.

Considerable strides have been announced recently in reducing cellulase costs, including the use of molecular biology to increase the enzyme efficiency. Molecular biology techniques can be used to increase the thermal stability of the enzyme, the pH tolerance, and catalytic efficiency on insoluble cellulosic substrates (Zhang et al. 2006). From the point of view of the process, improvements in synergistic action of the enzymes providing better mixing and matching and variations in reactor design have also been considered (Lee and Wolf 1988; Yu et al. 1999; Berlin et al. 2007).

It is noteworthy that the enzyme source has a major effect on the hydrolysis efficiency. Therefore, understanding the interaction between cellulases and pretreated biomass is vital to effectively develop low cost pretreatment and enzyme properties that can lead to competitive ethanol costs. However, the literature fails to identify key enzymes to assemble effective saccharolytic mixtures for each type of pretreated material. One often neglected subject is that the accessibility of enzymes to the substrate is critical during enzymatic hydrolysis; therefore, adsorption of cellulases on the substrate surface plays a relevant role (Martins et al. 2008). Cellulases that present productive adsorption on the substrate in a greater extent show an increase in the rate and extent of crystalline cellulose hydrolysis when compared with cellulases which adsorb less strongly.

The major enzymes involved in hydrolysis of lignocellulosic polysaccharides are modular proteins that contain, in addition to the catalytic module, a second module named carbohydrate binding domain (CBD). The CBD promotes a close contact between the substrate and the catalytic module of the enzyme, ensuring the correct orientation among them (Henrissat and Coutinho 1999; Wang et al. 2003; Borston et al. 2004). The presence of cellulose binding domains (CBD) in some cellulolytic enzymes explains, in part, the differences in adsorption ability of cellulases (Gilkes et al. 1991). The addition of CBD, derived from cellobiohydrolase II of *Trichoderma reesei* to the *Trichoderma harzianum* chitinase, allowed higher binding capability and resulted in increased hydrolytic activity toward insoluble substrates (Limon et al. 2001).

CBD is the second most important and wide-spread element of cellulases structure involved in cellulose transformation. In fact, CBD often plays the role of recognition factor, which is used by enzyme-producing microorganisms to address secreted polysaccharide hydrolases to the plant cell walls to be decomposed. The presence of the CBD in the cellulases enhances enzymatic hydrolysis, primarily of crystalline cellulose. It has been recognized that CBD increases the enzyme concentration on the surface of the solid substrate and perhaps perturbs the microfibril structure. The mechanism involves CBD binding to the planar face of cellulose microcrystal, whereas the catalytic domain (CD) binds to the hydrated cellulose chain end group. Release of the soluble sugar molecule and transformation of the productive CD into a new nonproductive CD complex opens the possibility for CBD to bind onto the surface again (Mosier et al. 1999).

In some instances, the role of CBD may be limited by the solely transportation of the enzyme to certain structural elements of native cellulose. This occurs in fungal cellobiose dehydrogenase (CDH), an extracellular flavohemeprotein that couples the process of cellulose and lignin breakdown (Henriksson et al. 1997). CDH has been found in various wood-degrading fungi (Henriksson et al. 2000). The enzyme does not split cellulose molecules, only oxidizes their reducing chain end to carboxylic groups, but presents a very high affinity for cellulose owing to the presence of CBD in its structure. The roles postulated for CDH include the generation of a hydroxyl radical. This is a consequence of its ability to reduce ferric iron providing ferrous iron in natural environments that in the presence of hydrogen peroxide give hydroxyl radical through the Fenton reaction (Hammel et al. 2002). This activity is very important because hydroxyl radical can start cellulose and/or lignin depolymerization. Another relevant role for CDH is to promote the cellobiohydrolase activity through the relief of end-product inhibition by oxidizing cellobiose. CDH also binds to the cellulose surface incrementally extending the amorphous region by pulling the molecule away from adjacent molecules at the periphery of crystalline regions. Oxidation of the reducing terminus of a cellulose molecule would presumably lock the terminal residue decreasing local crystallinity (Dumonceaux et al. 2001).

Cellulases have the ability to adsorb to lignin as well as cellulose. In some cases, up to 70% of the total enzyme added can be unproductively bound to lignin (Lu et al. 2002; Berlin et al. 2005a; Jorgensen and Olsson 2006). The cellulases adsorbed in lignin will not hydrolyze cellulose; they will thus be nonproductively bound. Once we know more about the adsorption mechanism, we might be able to control the adsorption to the productive sites. The large fraction of cellulases and hemicellulases unproductively bound to lignin emphasizes that methods aiming at lignin removal can be advantageous. Unproductive binding to lignin also prevents recycling of the enzymes, a strategy that has great impact on the economic viability of the process (Singh et al. 1991; Ramos et al. 1993; Lee et al. 1995; Gregg et al. 1998). To overcome this limitation, the surface of lignin can be covered with other proteins (albumin, for example), nonionic surfactants (Tween 20, for example), or polymers (polyethylene glycol, for example) which block hydrophobic sites in order to minimize undesirable adsorptions (Eriksson et al. 2002; Palonen et al.

2004; Yang and Wyman 2006; Borjesson et al. 2007). Pretreatments directed toward lignin solubilization with appropriate reagents (NaOH, for example) have also been proposed (Yang et al. 2002).

In theory, even the cellulase enzymes could be modified to avoid tightly binding to lignin, but keeping the ability of adsorption to cellulose. For example, Berlin et al. (2005b) showed that naturally occurring enzymes, with similar catalytic activities on model substrates, may differ in their interaction with lignin. Therefore, engineering or selection of enzymes with reduced affinity for lignin, so-called "weak-lignin binding enzymes," is a potential strategy to improve enzymes for hydrolysis of lignocellulosic substrates. It has been shown that increasing the hydrophilicity of cellulase by attaching a hydrophilic copolymer can increase desorption of cellulases from the substrate and consequently the conversion of substrate is improved (Park et al. 2002). However, the presence of polar functional groups (COOH, OH, CO) on both lignin and enzyme surfaces could also mediate electrostatic lignin-enzyme interactions.

The understanding of cellulolytic mechanisms can also be used to improve reaction efficiency in other fronts. For example, the enzymatic hydrolysis of cellulose to glucose is improved by up to 40% by the addition of β-glucosidases to the cellulase preparation (Berlin et al. 2007). Two other examples come from the discovery of the family of 61 protein and expansins.

Endoglucanase 61A is a putative metalloprotein with the ability to enhance digestion of lignocellulose. The endoglucanase 61A does not release significant amounts of soluble materials from cellulose or lignocellulose, but increases the hydrolysis of lignocellulose by cellulases (Dotson et al. 2007). Expansins have also been found to enhance cellulose accessibility by disrupting hydrogen bondings between plant cell wall polysaccharides. The expansins found in growing tissues of plant and plant-parasitic roundworm *Globodera rostochiensis* (Cosgrove 2000), the swollenin produced by *T. reesei* (Saloheimo et al. 2002), and the Gt factor produced by *Gloeophyllum trabeum* (Wang and Gao 2003) were shown to improve the hydrolysis efficiency when used as additives for cellulases during hydrolysis of cellulose. Furthermore, Han and Chen (2007) found that the crude cell wall extracts of corn stover could influence the cellulase activity, increasing conversion of cellulose to glucose and accelerating hydrolysis due to the presence of a corn stover protein named Zea h. The hydrogen bonds of the substrate may be weakened or ruptured by Zea h, as a result, the cellulose becomes more accessible to the cellulases.

Biomasses with hemicellulosic components may best be digested by novel enzyme mixtures containing a variety of hemicellulases comprising xylanases, mannanases, glucuronidases, and galactanases. A number of accessory enzymes could also be required, depending on the material being processed (Bhat and Hazlewood 2001). Therefore, the efficient degradation of hemicellulose requires the synergistic action of many enzymes. More importantly, hemicellulases facilitate cellulose hydrolysis by exposing the cellulose microfibrils, thus making them more accessible to the cellulases (Himmel et al. 2007a,b). Commercial development of hemicellulases for the ethanol industry is not as advanced as cellulases because

current commercial preparations have been primarily developed for dilute acid pretreated biomasses where hemicellulose has been removed before saccharification. However, with the development of nonacidic pretreatment methods (Jorgensen et al. 2007; Sánchez and Cardona 2008) or with the new generation of plants with reduced lignin content, the hemicellulose fraction could remain intact and the hemicellulases would be required. Current cellulases such as those from *T. reesei* tend to be poor in activity of hemicellulases and will not be adjusted for efficient conversion of hemicellulose-containing substrates into monomeric sugars (Gray et al. 2006). A new area to be explored in this subject is the use of "alkaline" xylanases from biobleaching processes, already currently used by some pulp and paper industries (Beg et al. 2001). In accordance with some recent studies (Oghren et al. 2007; Nguyen et al. 2008), the treatment of delignified plant tissues with hemicellulases increases the cell wall porosity not only because it causes an extensive hydrolysis of the hemicellulose, but also because it helps in the solubilization of residual lignin which eventually favors the action of cellulases.

From the point of view of using the enzymatic hydrolysates as a source of carbohydrates in fermentative processes, one can say that the use of hydrolysates with high concentrations of fermentable sugars can lead to high concentrations of products, which reduces the costs of separation and purification. However, performing the enzymatic hydrolysis in the presence of high concentrations of substrate (cellulose) presents two major limitations:

(1) Relatively low concentrations of glucose (1–14 mM) lead to inhibition of β-glucosidases, causing accumulation of cellobiose in the medium. Cellobiose, in turn, is a potent inhibitor of cellobiohydrolases, which leads to severe limitations in the hydrolysis of cellulose (Holtzapple et al. 1990; Tolan and Foody 1999; Decker et al. 2000). To overcome this limitation, the enzymatic saccharification (of cellulose in glucose) and fermentation (of glucose in ethanol) can be processed simultaneously (SSF), avoiding the accumulation of glucose in the medium (Alfani et al. 2000; Wingren et al. 2003).
(2) The use of cellulose concentrations exceeding 15% leads to excessive consistency, which hinders the mixing and increases the consumption of energy (Mohagheghi et al. 1992; Fan et al. 2003). To circumvent this problem, the SSF can be operated in fed-batch mode. The hydrolysis is initiated with a substrate concentration of less than 10% and, as cellulose is liquefied and consistency reduced, more substrate is added to the reaction tank (Varga et al. 2004; Rudolf et al. 2005).

7 Final Remarks

Although much is known about the chemical changes caused in lignocellulosic materials by different pretreatments, little has been reported on the physical and topochemical changes occurring during those treatments. It is our particular view

that breakthroughs in this area must be accompanied by ultrastructural and molecular investigations of the lignocellulosic material. Taking the fibrous and lignified cell wall characteristics of the lignocellulosic materials, it is clear that lignin represents one of the main hindrances for ethanol production from lignocellulosic biomass. Lignin and hemicellulose embed the cellulose, thereby offering protection against microbial, enzymatic and even chemical degradation. Furthermore, lignin is able to form covalent bonds to some hemicelluloses. In the same direction, the presence of hemicelluloses has long been neglected when considering the performance of enzyme mixtures for hydrolysis of cellulose. Hemicellulose is found in close association with the cellulose microfibrils together with lignin, and both fractions limit the access of the cellulases to the cellulose backbone. Similarly to lignin, the type and distribution of hemicellulose depends on the vegetable species. Additionally, the recalcitrant nature of the residual lignin is due in part to the presence of associated xylan. Xylanase treatment of plant tissues depleted on lignin could increase the cell wall porosity helping on solubilization of residual lignin bound to xylans and favoring the subsequent action of cellulases (Oghren et al. 2007). It is expected that the use of xylanase will also cause extensive hydrolysis of xylans that are not associated with residual lignin. The ability of accessory enzymes to debranch hemicellulose side groups that may interact with residual lignin holds promise for increases in the cell wall porosity owing to side delignification effects, as shown in a recent study assessing the potential of a feruloyl esterase from *Aspegillus niger* to bleach pulps (Nguyen et al. 2008). The use of expansins to split hydrogen bonding in polysaccharides could also be of great interest.

In the ethanol production from cellulosic materials, all the factors hindering the access of hydrolytic enzymes to the polysaccharides would increase the production cost, once the enzyme is partially consumed during the process (Cantarella et al. 2004; Panagiotou and Olsson 2007). It is remarkable that the removal of significant amounts of lignin in steam-exploded softwood by using alkaline extraction (Ramos et al. 1992) or in untreated wood flour by using ionic liquids (Lee et al. 2009) significantly improved the substrate digestibility. Furthermore, due to the lower lignin content, enzyme loadings can be considerably reduced. However, extensive lignin removals by pretreatments add cost to the processes. Although enzyme price has decreased due to intensive research to improve its production, enzyme load during cellulose hydrolysis should be minimized because it also increases the cellulosic-ethanol production costs.

In the context of the worldwide advances obtained in the area of enzymatic conversion of cellulosic substrates, it is our view that additional research efforts should be directed to advance the knowledge related to the lignin hindrance for enzymatic hydrolysis of lignified cells. For this, a topochemical approach able to provide a complete view of lignin distribution in the cell walls and its effect on subsequent enzymatic hydrolysis of polysaccharides is desirable. Considering that plants down regulated on lignin biosynthesis are upcoming, future processes for cellulosic-ethanol production seem to be focused on milder pretreatments followed by efficient enzymatic hydrolysis of the entire polysaccharide fraction.

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Chapter 6
Enzymology of Plant Cell Wall Breakdown: An Update

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1 Introduction

The continued usage of fossil fuels to support the world economy has significant negative environmental consequences. Thus, there is great interest in utilizing renewable sources to supply our fuel and chemical feedstock needs. The plant cell wall represents half of the organic carbon in the biosphere, being a plentiful renewable substrate and, therefore, a potential candidate to displace a large fraction of products currently derived from fossil fuels (Lee et al. 2008). Its physiological functions during plant life include growth, intercellular communication, defense against pathogen attack, mechanical resistance and interaction with the environment. Even so, there are many organisms in nature that can efficiently degrade the cell wall and use by-products of its degradation for nutrition (Minic and Jouanin 2006).

Lignocellulose, the most abundant biomaterial in nature, is mainly composed by cellulose, hemicelluloses and lignin (Juhász et al. 2005), forming plant cell walls in different proportions. Its predominant polysaccharide is cellulose, followed by hemicellulose, which usually forms hydrogen bonds with the first, as well as with other hemicelluloses, helping in the stabilization of the cell wall matrix and making it water insoluble (Himmel et al. 1999). The study of the enzymes involved in cell wall breakdown has been intense since the early 1950s (Reese 1976; Coughlan 1985; Mandels 1985). The sugars released during the enzyme acting have many applications in different industries.

Hydrolytic enzymes such as cellulases, xylanases, and mannanases contribute to the degradation of the carbohydrate moieties, while oxidative enzymes such as laccases, lignin peroxidase, and manganese peroxidase, in combination with low-molecular-weight mediators, have been shown to be involved in lignin biodegradation (Manfield et al. 1997).

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The plant cell wall complexity and heterogeneity requires a large spectrum of exo- and endo-enzymatic activities (Minic and Jouanin 2006). The degradation of lignocellulosic materials to monomeric sugars has great importance, since fermentable sugars can be used as raw materials in a number of biotechnological production processes, including the ethanol production (Lawford and Rousseau 2003). As matter of fact, two types of enzyme are required to carry out the breaking down of plant cell wall polysaccharides. The exohydrolases are responsible for acting on the terminal glycosidic linkages and liberate terminal monosaccharide units, while endohydrolases cleaves internal glycosidic linkages at random or at specific positions, usually internally (Coughlan 1992). The plant cell wall structure is a good environment to induce substrate promiscuity (Siqueira and Filho 2010). In this case, enzyme systems with high substrate promiscuity act in synergism with enzymes with strict substrate specificity, leading to a more efficiency in the catalytic process. Within this context, enzymes that cut specific sites in the plant cell wall are also important tools for understanding the structure and function of cell wall. Thus, it is clear that one must have a thorough understanding of the enzymatic systems required for the breaking down of each of the components of lignocellulose structure in plant cell wall.

The progress in biotechnology of cellulases and related enzymes attracts worldwide attention. Currently, cellulases, hemicellulases and pectinases are widely used in biofuel, food, brewery and wine, animal feed, textile and laundry, paper and pulp industries as well as in research and development. Some of these applications prefer one or two selected enzymatic components, while others require mixtures of all of them for maximum benefit (Bhat 2000). This chapter aims to overview the main studied enzymes involved in the cell wall degradation. The knowledge of the potential of these enzymes raises the possibility of development of new technologies, including the green ones.

2 Cellulases

Many enzymes are involved in the degradation of the polymers that compose lignocellulose (Ward and Moo-Young 1989). Its predominant polysaccharide is cellulose, which is a crystalline matrix of linear β-(1,4)-D-glucan chains (DGlc) (Himmel et al. 1999).

In the middle of the twentieth century began the discussions about the complexity of the natural cellulolytic enzymes and their different abilities to degrade cellulose. It was speculated that there were three types of enzyme activities involved in hydrolyzing cellulose: C_1, which would convert crystalline cellulose to amorphous, C_x, which would hydrolyze amorphous cellulose to cellobiose, and β-glucosidase, which would hydrolyze the soluble cellobiose to glucose (Tolan and Foody 1999).

The current opinion about cellulases diversity and action still agrees with the synergistic and coordinate attack of cellulose for a complex of enzymes,

facilitating the degradation of the polymer (Lynd et al. 2002). These enzymes are described in terms of three major classes of cellulases. The endoglucanases (EC 3.2.1.4, EG) act randomly on soluble and insoluble cellulose chains. The exoglucanases, which include cellobiohydrolases (EC 3.2.1.91, CBHs), act processively to preferentially liberate cellobiose (and glucose in some cases) from the reducing (CBHII) and nonreducing (CBHI) ends of the cellulose chain. The β-glucosidases (EC 3.2.1.21) liberate D-glucose from cellobiose and exoglucosidases (Himmel et al. 1999; Tolan and Foody 1999). Among the studied microorganisms, fungi are most active against natural polymers (Sohail et al. 2009), being capable of producing different amounts of each type of cellulase (Picart et al. 2007), which act sinergistically.

Almost all commercial cellulases obtained by submerged fermentation are produced by the fungi *Trichoderma*, *Humicola*, *Aspergillus* and *Penicillium* (Tolan and Foody 1999), and proteins from *Trichoderma* and *Aspergillus* involved in the transcriptional regulation of the genes encoding cellulases and hemicellulases have already been identified (de Vries and Visser 2001; Mach and Zeilinger 2003). The inducer molecules produced during degradation of the lignocellulosic material regulate positively the expression of these enzymes, e.g., cellobiose, D-xylose and L-arabinose (de Vries 2003). However, essential differences between the fungi do exist (Jorgensen et al. 2005). Sophorose, for example, is an effective inducer of cellulase genes and some xylanase genes in *Trichoderma* (Mach and Zeilinger 2003) but this disaccharide does not function as an inducer of an endoglucanase gene in *Penicillium janthinellum,* having no effect on the expression of several cellulolytic genes in *A. niger* (Gielkens et al. 1999). In general, cellulase is inhibited by its end products, cellobiose and glucose. Its action is also inhibited or inactivated by several classes of compounds, including strong oxidants or reducing agents, metal ions, salts, solvents, and surfactants (Tolan and Foody 1999).

Most cellulases are modular enzymes containing two or more discrete modules, described as catalytic and carbohydrate-binding modules (CBMs). They are connected to each other via a linker sequence (Mosier et al. 1999; Arai et al. 2003). Catalytic modules, which are engaged in the hydrolysis of cellulose, are classified in 14 groups in glycoside hydrolase (GH) families on the basis of amino acid sequence similarities (http://afmb.cnrs-mrs.fr/cazy/CAZY/index.html). On the other hand, CBMs, many of which bind preferentially to cellulose, are also classified into 30 families on the basis of amino acid sequence similarities (http://afmb.cnrs-mrs.fr/cazy/CAZY/index.html). It is thought that a CBM in a cellulase molecule enhances the hydrolytic activity of a catalytic domain adjacent to the CBM by increasing the enzyme concentration on the surface of an insoluble substrate or by supplying the catalytic module with a more easily degradable substrate, i.e., amorphous cellulose. Cellulases can provide huge benefits in the utilization of biomass in the long term because of the possible high glucose yields and the opportunity to apply the modern tools of biotechnology to reduce costs (Himmel et al. 1999).

2.1 Cellobiose Dehydrogenase

Cellobiose dehydrogenase (EC 1.1.99.18) is produced extracellularly by a number of wood- and cellulose-degrading fungi when grown on cellulose. It oxidizes the reducing end of cellobiose and cellooligosaccharides to their corresponding 1,5-lactones, which are subsequently hydrolyzed to carboxylic acids in aqueous environments. In addition to the cellooligosaccharides, the presumed natural substrates, CDH oxidizes very few other sugars, the most efficient substrates being β-1,4-linked disaccharides with a β-glucose moiety at their reducing end (Baminger et al. 2001).

CDH is a monomeric enzyme consisting of two prosthetic groups, a heme and a flavin adenine dinucleotide moiety (hemoflavoenzyme). The latter domain is directly involved in both the oxidative and reductive half-reactions and the reduced enzyme is reoxidized by different electron such as cytochrome *c* and Fe^{3+}. These two domains are linked by a protease-sensitive region (Manfield et al. 1997; Baminger et al. 2001).

The in vivo function of CDH is not fully understood. CDH is not an essential component of the lignocellulose-degrading enzyme complex but can enhance both cellulose and lignin degradation. CDH also could have a protective function since it can reduce quinones, one of the major antimicrobial systems used by plants (Baminger et al. 2001).

3 Hemicellulases

Hemicelluloses are structural polysaccharides of the plant cell wall in close association with cellulose and lignin, forming the lignocellulosic biomass (Moreira and Filho 2008). Hemicelluloses are heterogeneous polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose), and sugar acids (glucuronic acid) Unlike cellulose, hemicelluloses are not chemically homogeneous. Hardwood hemicelluloses contain mostly xylans, whereas softwood hemicelluloses show a high percent of glucomannans (Saha 2003). Hemicelluloses are classified as arabinoxylan, acetylglucuronoxilan, xylan, xyloglucan, arabinan, arabinogalactn, galactomannan, glucomann and galactoglucomannan.

The utilization of hemicellulosic sugars is essential for efficient and cost-effective conversion of lignocelluloses material to biofuel (ethanol). In 2002, over two billion gallons of ethanol was produced, mainly by fermenting corn starch (Saha 2003).

Hemicellulases are frequently classified according to their action on distinct substrates. Xylan is the main carbohydrate found in hemicellulose. Its complete degradation requires the cooperative action of a variety of hydrolytic enzymes, such as endo-1,4-β-xylanase and 1,4-β-xylosidase (Pérez et al. 2002). Besides, hemicellulose degradation needs accessory enzymes as xylan esterases, α-L-arabinofuranosidases,

ferulic and *p*-coumaric esterases, and α-4-*O*-methyl glucuronidases acting synergistically to efficiently hydrolyze wood xylans and mannans.

3.1 Xylanases

The structure of xylans found in cell walls of plants can differ greatly depending on their origin, but they always contain a β-1,4-linked D-xylose backbone (de Vries and Visser 2001). Owing to its heterogeneous structure, xylan degradation needs not just one enzyme, but an enzyme complex. Xylanases are produced mainly by microorganisms and take part in the breakdown of plant cell walls, along with other enzymes that also hydrolyze polysaccharides (Polizeli et al. 2005).

Xylan are the major constituent of the hemicelluloses with linear or branched backbone of β-1,4-linked xylopyranose units respectively (Polizeli et al. 2005). β-Xylanases (EC 3.2.1.8) and side-chain enzymes are required for an efficient breaking down of xylan to fermentable sugar or oligosaccharides. Side-chain enzymes remove substituents that are attached at various points on xylan, creating more sites for subsequent enzymatic hydrolysis of xylan. Endo-1,4-β-xylanase (EC 3.2.1.8) is the crucial enzyme for xylan depolymerization, which hydrolyses β-1,4-bonds between D-xylose residues in the main chain generating nonsubstituents or branched xylooligossacharides. Morevover, α-L-arabinofuranosidase, α-glucuronidase, acetyl (xylan) esterase, ferulic and *p*-coumaric acid esterase have cooperative functions in the complete degradation of xylan (Biely et al. 1997).

Endo-1,4-β-xylanase cleaves the glycosidic bonds in the xylan backbone, bringing about a reduction in the degree of polymerization of the substrate. Initially, the main hydrolysis products are β-D-xylopyranosyl oligomers, but at a later stage, small molecules such as mono-, di- and trisaccharides of β-D-xylopyranosyl may be produced. Acetylxylan esterase (EC 3.1.1.6) removes the *O*-acetyl groups from positions 2 and/or 3 on the β-D-xylopyranosyl residues of acetyl xylan. α-Glucuronidase (EC 3.2.1.131) hydrolyzes the α-1,2 bonds between the glucuronic acid residues and β-D-xylopyranosyl backbone units found in glucuronoxylan (Polizeli et al. 2005)

3.2 Arabinofuranosidases

Lately, much of attention has been given to α-L-arabinofuranosidases (α-L-AFases EC 3.2.1.55). In many plants, xylan backbone is substituted by different side chains with L-arabinose, D-galactose, acetyl, feruloyl, *p*-coumaroyl and glucuronic residues (de Vries and Visser 2001; Adams et al. 2004). There can be differences in the composition and in the frequency of the side chains of xylans of different types of plants (Saha 2000; de Vries and Visser 2001). The α-L-arabinofuranose (α-L-Ara*f*) residues are part of many plant cell wall polyssacharides, including arabinoxylan,

arabinogalactan, and arabinan. In arabinans they are 1,5-linked to the backbone, as well as 1,2- and 1,3-linked as side chains. They are associated with pectic substances in a great variety of sources, e.g., sugar beet and citrus pectins. In arabinogalactans they are found as 1,3- and 1,6-linked side chains (de Wet et al. 2008). Arabinoxylan is the major component of the cell wall of softwoods, as well as grasses and cereals. It consists of a backbone of 1,4-linked β-xylopyranosyl units that have α-L-Ara*f* substituents at the *O*-2 and *O*-3 positions (Bacic et al. 1988).

The L-arabinosyl residues distributed as side chains restrict the enzymatic hydrolysis of hemicelluloses and pectins (Saha 2000; Rahman et al. 2003). The α-L-AFases are accessory enzymes that cleave α-L-arabinofuranosidic linkages at the nonreducing ends of arabinose-containing polysaccharides and play an integral role in the biodegradation of hemicellulose (Saha 2000). The action of these enzymes accelerates the hydrolysis of the glycosidic bonds by more than 10^7 fold, making them one of the most efficient catalysts known (Rye and Withers 2000; Shallom et al. 2002). They act synergistically with other hemicellulases and pectic enzymes for the complete hydrolysis of hemicelluloses and pectins (Spagna et al. 1998).

The α-L-AFases exhibit wide substrate specificity for not distinguishing the saccharide link to the arabinofuranosyl moiety (Rahman et al. 2003). They catalyze the hydrolysis of terminal α-L-1,2-, α-L-1,3- and α-L-1,5-arabinofuranosyl residues (Saha and Bothast 1998; Saha 2000), but also residues from pectic, homo and hemicellulosic polysaccharides (branched arabinans, debranched arabinans), heteropolysaccharides (arabinogalactans, arabinoxylans, arabinoxyloglucans, glucuronoarabiloxylans, etc.) and different glycoconjugates (Beldman et al. 1997; Sozzi et al. 2002). Based on primary sequence, α-L-AFases are grouped into GH families 43, 51, 54, and 62, each one with unique substrate specificity (de Wet et al. 2008). The recently described crystal structure of a α-L-AFase (GH 54 family) from *Aspergillus kawachii* (Miyanaga et al. 2004) and whose active site in the catalytic domain similarly accommodates the -1 α-L-Ara*f* in a shallow surface pocket, explains its ability to hydrolyze a variety of sugars, linked from different positions to *O*-1 of the α-L-Ara*f*.

The action of α-L-AFases alone or in combination with other lignocellulose-degrading enzymes represents a promising biotechnological tool as alternatives to some of the existing chemical technologies such as chlorination in pulp and paper industry, synthesis of oligosaccharides and pretreatment of lignocelluloses for bioethanol production (Numan and Bhosle 2006).

3.3 Feruloyl esterase

Feruloyl esterases or ferulic acid esterases (FAEs, E.C. 3.1.1.73) are members of the carboxylic ester hydrolases subclass of enzymes, which are responsible for cleaving the ester-link between the polysaccharides in the main chain of xylans or pectins, and monomeric or dimeric ferulic acid. These polysaccharides which

complex together with macrocolecular complexes like lignin and cellulose, modulate the plant cell wall material. To date, feruloyl esterases prove to be the key in the enzymatic synthesis of phenolic sugar esters (Vafiadi et al. 2006). Ferulic acid (FA) ester-links arabinose, one of arabinoxylan's side chain types, to lignin (Ralph et al. 1995). Since FAEs hydrolyze the bond between arabinose and FA, they may release the covalently bound lignin from hemicelluloses and aid in the degradation of plant cell walls. Moreover, cross-links through diferulic bridges are found both in heteroxylans and pectin tissues thus playing an important role in the structure of nonlignified cell walls (Saulnier and Thibault 1999).

FAEs occur as single catalytic modules and also as a part of multimodular protein structures. Some enzymes contain cellulose-binding modules (CBM) and some others are part of multimodular complex, such as cellulosomes (Blum et al. 2000). The fusion of a CBM to a catalytic domain improves their catalytic efficiency (reviewed by Vafiadi et al. 2006).

Since the 1990s, more than 30 FAEs have been purified and characterized from a wide range of microorganisms, including bacteria and fungi (Faulds et al. 1995; de Vries et al. 2002). Many enzymes from the genus *Aspergillus*, *Clostridium* and *Penicillium* have been studied (Topakas et al. 2007). The purified FAEs show significant variations in physical characteristics such as molecular weight, isoelectric point and optimum hydrolytic reaction conditions (Topakas et al. 2007).

The nomenclature of FAEs follows both the source of the enzyme and the type of the esterase (Topakas et al. 2007). FAEs are subclassified into four types (A–D) and indicate evolutionary relationship between feruloyl esterases, acetyl xylan esterases and certain lipases. The four functional classes also take into account substrate specificities against synthetic methyl esters of hydroxycinnamic acids, growth substrate requirements of the microorganisms and protein sequence identity (Crépin et al. 2004). Each feruloyl esterase has its own specificity with regard to the release of specific cinnamic acids. Two major enzymes were purified from *Aspergillus niger* and classified as types A and C. The first does not hydrolyze methyl ester of caffeic acid but is able to release diferulic compounds. The second hydrolyzes the four methyl esters of hidroxycinnamic acids generally used as model substrates but not diferulic compounds (Benoit et al. 2006).

It is extremely common for esterases to act on a broad range of substrates. Type A FAE shows preference for the phenolic moiety of the substrate containing methoxy substitutions, especially at meta-position(s), as occur in ferulic and sinapinic acids. Type B FAE shows complementar activity to type A esterases, preferring substrates containing one or two hydroxyl substitutions as found in *p*-coumaric or caffeic acid. In contrast to type B, type A FAE seems to prefer hydrophobic substrates with bulky substituents on the benzene ring (Kroon et al. 1997; Topakas et al. 2005). In any case, the enzyme requires a certain distance between the aromatic ring of substrates and the ester bond for the catalytic activity (Kroon et al. 1997; Vafiadi et al. 2006).

Studies with an inactive mutant of the *A. niger* type A feruloyl esterase (AnFaeA) complexed with a feruloylated trisaccharide demonstrated that tight binding of the

carbohydrate is not required for catalysis (Faulds et al. 2005). Also Type C feruloyl esterase from *Sporotrichum thermophile* (StFaeC) in nonconventional media seems to be able to sterify a broad spectrum of sugars, showing specificity only on the ferulic moiety (Vafiadi et al. 2005).

As phenolic acid sugar esters have presented antitumoric activity, they could be applied in the formulation of antimicrobial, antiviral and/or anti-inflammatory agents (Vafiadi et al. 2006). FAEs could also be used in pulp and paper processing and as animal feed additives to facilitate nutrient assimilation. FAEs play a vital role in the delignification and depolymerization of polysaccharide chains as the cross-linking through the FA ester bond substantially increases the recalcitrance of biomass and the resistance to enzymatic hydrolysis. Besides, FA released from plant cell wall by the action of FAEs is an effective natural antioxidant with potential applications in the pharmaceutical and food industries (reviewed by Topakas et al. 2007).

3.4 Coumaroyl Esterase

Lignin plays a critical role in biomass utilization. Lignins are macromolecular complexes composed of three phenylpropanoid monomers, *p*-coumaryl, coniferyl, and sinapyl alcohols coupled through dehydrogenative polymerization via combinatorial radical coupling reactions. In many crops, lignins may be acylated by various acids (Ralph et al. 2004), though the process and function have not been resolved yet. The enzyme responsible for this acylation reaction is *p*-coumaroyl transferase (pCAT).

The anaerobic rumen fungi are known preferentially to colonize and degrade lignified cell walls, which are the least biodegradable walls (Akin et al. 1983). These fungi were found to produce high levels of both *p*-coumaroyl and feruloyl esterase (Borneman et al. 1990). Borneman et al. (1991) proposed that *p*-coumaroyl and feruloyl esterases may provide the anaerobic fungi with an advantage in the degradation of phenolic-containing plant cell walls, providing an ecological niche for the fungi in the rumen.

Studies suggest that ester linkages between phenolic moieties and arabinoxylans prevent the enzymatic hydrolysis and limit utilization by bacteria of otherwise available carbohydrates (Hespell and O'Bryan 1992). Therefore, possession of feruloyl and *p*-coumaroyl esterases, coupled with the penetrative ability given by fungal rhizoids or rhizomicelia, provides a mechanism for the degradation and subsequent utilization of the phenolic ester-linked carbohydrates in plant cell walls (Borneman et al. 1992).

The xylans of gramineaceous cell walls are often highly substituted with arabinosyl residues, many of which are esterified with *p*-coumaroyl and feruloyl groups (Hartley and Ford 1989). There is evidence that these esterified phenolic groups dimerize in vivo either by oxidative coupling, forming dehydrodiferulic acid (Ishii 1991), or by photodimerization in sunlight, forming a series of substituted truxillic and truxinic acid cross-links (Hartley et al. 1990). Evidence also suggests that feruloyl and

p-coumaroyl groups form bonds between the xylan heteropolymer and lignin (Scalbert et al. 1985), possibly limitating cell wall biodegradation (Hartley et al. 1990). Furthermore, the esterification of feruloyl and *p*-coumaroyl groups to arabinoxylans has been shown to limit the release of cell wall pentoses (Hespell and O'Bryan 1992). *p*-Coumaric acid, as a phenolic acid, is an antioxidant which is implicated for the prevention of pathologies, such as colon cancer and cardiovascular diseases (reviewed by Liu et al. 2006).

3.5 Xyloglucanases

The xyloglucan (XG) family of polysaccharides receives considerable attention due to the key structural role these matrix polymers play in the cell walls of higher plants. XGs are major components of the primary wall of all dicots and some monocots where they bridge paracrystalline cellulose microfibrils by surface adsorption and chain intercalation thereby modulating wall mechanical properties and affecting morphology (Baumann et al. 2007). XG acts as the interface between cellulose and other polysaccharides in the primary cell wall and seeds of mono and dicotyledons. They are composed of a backbone of 1,4-linked β-D-glucopyranosyl units with side chains of D-xylosyl residues attached by α-link to *O*-6 of some of the glucosyl residues (Couglan et al. 1993).

Enzymes involved in the modification of XG structure are XG specific endoglucanases including XG endo-transglycosylase/hydrolases (XTH, 2.4.1.2070, a XG-active β-D-galactosidase (3.2.1.23), a XG specific α-L-fucosidase (3.2.1.51/3.2.1.63) and a XG oligosaccharide-specific α-D-xylosidase (3.2.1.37) (Minic and Jouanin 2006). XTHs appear to play a major role in plant growth and development because they are probably involved in the construction and restructuration of the plant cell wall (Fry 2003). It cleaves a donor XG to release a smaller XG, with a reducing end in the first step, and then transfers the remainder to the acceptor, an oligosaccharide. Finally, α-D-xylosidase cleaves specifically the α-xylosyl residue attached to the glucose residue of the xyloglucanoligosaccharide (Minic and Jouanin 2006). XTHs in plants are assigned to family 16 of GHs. The structure and organization of the XTH gene family were analyzed in *Arabidopsis* and 33 open reading frames (ORFs) potentially encoding XTH proteins that were divided into three major phylogenetic groups or subfamilies were identified (Rose et al. 2002).

3.6 Mannanases

β-mannanases have been classified within families 5 and 26 of GHs (Henrissat and Bairoch 1993). They are important enzymes for the enzymatic degradation of hemicelluloses from softwood. The mannan-degrading enzymes are composed of β-mannanase (1,4-β-D-mannan mannohydrolase, EC 3.2.178), β-mannosidase

(1,4-β-D-mannopyranoside hydrolase, EC 3.2.1.25), and β-glucosidase (1,4-β-D-glucoside glucohydrolase, EC 3.2.1.21). Additional enzymes such as acetyl mannan esterase (EC 3.1.1.6) and α-galactosidase (1,6-α-D-galactoside galactohydrolase, EC 3.2.1.22) are required to remove side-chain substituents that might be attached at various points on the mannan structure, creating more sites for subsequent enzymic hydrolysis (Filho 1998). β-Mannanase, an endo-type enzyme, is responsible for the cleavage of β-1,4-linked internal linkages of the mannan backbone randomly to produce new chain ends. In nature, endo-1,4-β-mannanase cleaves the main chain of galactomannan, yielding principally oligosaccharides and mannobiose. Subsequently, 1,4-β-mannosidase (EC 3.2.1.25) produces mannose (Xu et al. 2002; Moreira and Filho 2008). The degradation of galactomannan and galactoglucomannan by β-mannanase is greatly affected by the extent and pattern of substitution of the mannan backbone. β-Mannosidase, an exo-type enzyme, cleaves β-1,4-linked mannosides, releasing mannose from the nonreducing end of mannans and mannooligosaccharides. β-Glucosidase, an exo-type enzyme, hydrolyze 1,4-β-D-glucopyranose at the nonreducing end of the oligosaccharides released from glucomannan and galactoglucomannan by β-mannanase. α-Galactosidase, a debranching enzyme, catalyzes the hydrolysis of α-1,6-linked D-galactopyranosyl side chains of galactomannan and galactoglucomannan. Acetyl mannan esterase, a debranching enzyme, releases acetyl groups from galactoglucomannan (Moreira and Filho 2008). The mannan structure affords the synergistic actions of a variety of main- and side-chain-cleaving enzymes (Couglan et al. 1993). These enzymes have ability to degrade several substrates; this might be explained by the presence of multiple catalytic or binding sites where each substrate is being hydrolyzed at a different site in the enzyme (Gübitz et al. 1996; Magalhães and Milagres 2009).

4 Glucuronidases

Glucuronic acid residues and their 4-*O*-methyl ethers can be removed from the xylan backbone by α-glucuronidases (EC 3.2.1.131). The activity of this enzyme has been detected in a large number of fungal and bacterial culture filtrates, but α-glucuronidases have been purified from only a small number of organisms. The enzyme is active mainly on small xylooligomers and therefore is dependent on the action of endoxylanases. α-Glucuronidases have the highest activity against oligosaccharides, whereas only low or no activity is observed against polymeric substrates (de Vries and Visser 2001). Most of the α-glucuronidases will release glucuronic acid only if the chemical moiety is located on the terminal xylose on the nonreducing end of xylo-oligosaccharides. However, there have been reports of α-glucuronidase enzymes that hydrolyze internal glucuronic acid residues from glucuronoxylan (Lee et al. 2008). Synergy between α-glucuronidases and endoxylanases and between α-glucuronidases and β-xylosidase has been reported (de Vries and Visser 2001; Lee et al. 2008). Despite the important nature of the

glucuronic acid substituent, there are very few known α-glucuronidase-encoding genes (<40). This situation is caused in part by the lack of a high-throughput activity assay (Lee et al. 2008).

5 Pectinases

Pectinases are one of the upcoming enzymes of the commercial sector, especially the juice and food industry and in the paper and pulp industry (Jayani et al. 2005). Pectinases are a heterogeneous group of related enzymes that hydrolyze the pectic substances, present mostly in plants. Pectinolytic enzymes are widely distributed in higher plants and microorganisms. They are of prime importance for plants as they help in cell wall extension and softening of some plant tissues during maturation and storage (Jayani et al. 2005; Uenojo and Pastore 2007).

It has been reported that microbial pectinases account for 25% of the global food enzymes sales. Almost all the commercial preparations of pectinases are produced from fungal sources. *A. niger* is the most commonly used fungal species for industrial production of pectinolytic enzymes (Jayani et al. 2005).

Pectinolytic enzymes can be divided in three major groups based in the attack to galacturonic backbone, preference to the substrate (pectin, pectic acid or protopectin) (Uenojo and Pastore 2007)

5.1 Protopectinases

Protopectinases or pectinosidases degrade the insoluble protopectin and give rise to highly polymerized soluble pectin and can be classified into two types, on the basis of their reaction mechanism. A-type of protopectinases (PPase-A) react with the inner site, i.e., the polygalacturonic acid region of protopectin, whereas B-type protopectinases (PPase-B) react on the outer site, i.e., on the polysaccharide chains that may connect the polygalacturonic acid chain and cell wall constituents (Jayani et al. 2005; Uenojo and Pastore 2007).

5.2 Polygalacturonase

Polygalacturonases (PGases) are the pectinolytic enzymes that catalyze the hydrolytic cleavage of the polygalacturonic acid (1,4-α-D-galacturonide) chain with the introduction of water across the oxygen bridge. They are the most extensively studied among the family of pectinolytic enzymes. The PGases involved in the hydrolysis of pectic substances are:

Endo-PGase (E.C. 3.2.1.15) with random hydrolysis.
Endo-PGase (E.C. 3.2.1.67) with sequencial hydrolysis (Kashyap et al. 2001; Jayani et al. 2005; Uenojo and Pastore 2007).

5.3 Lyases

Lyases (or transeliminases) perform nonhydrolytic breakdown of pectates or pectinates, characterized by a trans-eliminative split of the pectic polymer. The lyases break the glycosidic linkages at C-4 and simultaneously eliminate H from C-5, producing a Δ 4:5 unsaturated (Jayani et al. 2005). Lyases can be classified into following types on the basis of the pattern of action and the substrate acted upon by them:

Endopolygalacturonate lyase (EndoPGL, E.C. 4.2.2.2) catalizes random cleavage of α-1,4-glicosidic linkage in pectic acid.
Exopolygalacturonate lyase (ExoPGL, E.C. 4.2.2.9) catalizes sequencial cleavage of α-1,4-glicosidic linkage in pectic acid.
Endopolymethylgalacturonate lyase (EndoPMGL E.C. 4.2.2.10), catalizes random cleavage of α-1,4-glycosidic linkages in pectin.
Exopolymethylgalacturonate lyase (exo PMGL) catalizes stepwise breakdown of pectin by trans-eliminative cleavage (Kashyap et al. 2001; Jayani et al. 2005).

6 Swollenin

Swollenin, a protein with sequence similarity to the plant expansins, breaks down hydrogen bonds between cellulose microfibrils or cellulose and other cells (Cosgrove 2000). Unlike plant expansins, swollenin has a bimodular structure composed of N-terminal fungal type carbohydrate binding domain, connected by a linker region to the plant expansin homologous domain with homology to the group 1 grass pollen allergens (pfam 01357). This modular structure is typical of fungal cellulases and some hemicellulases that contain one or several CBMs to target the catalytic module close to the substrate (Levasseur et al. 2006; Brotman et al. 2008)

This protein was first isolated and characterized from the saprophytic cellulolytic fungus *Trichoderma reesei,* and was named swollenin due to its ability to swell cotton (*Gossypium hirsutum*) fibers without producing detectable amounts of reducing sugars, pointing to potential application in promoting lignocelluloses enzymatic degradation (Saloheimo et al. 2002; Brotman et al. 2008; Yao et al. 2008). Moreover swollenin has been found to weaken filter paper (Yao et al. 2008). The protein also contains regions similar to mammalian fibronectin type III repeats, found for the first time in a fungal protein. The swollenin gene is regulated in a largely similar manner as the *T. reesei* cellulose genes (Saloheimo et al. 2002).

In the fungal database, proteins sharing similar domain structure to swollenin, a CBD domain linked to an expansin-like domain, are present only in *Aspergillus fumigatus* and *Neosartorya fischeri* (Brotman et al. 2008). Brotman et al. (2008) examined *Trichoderma* mutants with overexpression or silencing of the swollenin gene, providing evidence that this protein remarkably increases fungus plant root colonization efficiency and that the CBD domain is indispensable for protein full activity in vivo.

7 GH 61

The extensive variety of stereochemistry of carbohydrates is paralleled by a large multiplicity of the enzymes involved in their metabolism. Glycosyl hydrolases (EC 3.2.1.x) are key enzymes of carbohydrate metabolism that are found in the three major kingdoms (archaebacteria, eubacteria and eukaryotes). Heritable deficiencies in glycosyl hydrolases, for example lactose intolerance or mucopolysaccharidosis are among the most frequent genetically based syndromes in man (Henrissat 1991).

Glycosyl hydrolases have been classified into families of related sequences using hydrophobic cluster analysis (Henrissat and Bairoch 1993). The glycosyl hydrolase (GH) family 61 is a long-recognized class of proteins, with little known about the activity, mechanism or function. GHs have been classified into more than 100 families (Karkehabadi et al. 2008; Koseki et al. 2008). GH family 61 includes a endoglucanase from *Aspergillus kawachii* (AkCel61) that is a modular enzyme consisting of a catalytic domain and a carbohydrate-binding module belonging to family 1 (CBM1) that are connected by a Ser-Thr linker region longer than 100 amino acids (Koseki et al. 2008). The family 61 endoglucanases of GHs have been described only in fungi (http://afmb.cnrs-mrs.fr/CAZY). The endoglucanases EGIV from *Trichoderma reesei* (Saloheiomo et al. 1997) and CEL1 from *Agaricus bisporus* (Raguz et al. 1992) also belong to GH 61 family. Cel61A from the filamentous fungus *Hypocrea jecorina* is reported to act on microcrystalline cellulose and cellulose derivative substrates, including carboxymethyl cellulose and phosphoric acid-swollen cellulose (Karlsson et al. 2001; Karkehabadi et al. 2008). In addition, this enzyme is also active against β-glucan, lichenan and cellooligosaccharides. Other examples of endoglucanases in GH 61 family are from *Cryptococcus neoformans* (Chang and Kwon-Chung 1998 – cited by Koseki et al. 2008), *Phanerochaete chrysosporium* (Wymelenberg et al. 2002 – cited by Koseki et al. 2008), *Cochliobolus heterostrophus* (Lev and Horwitz 2003 – cited by Koseki et al. 2008), *Aspergillus kawachii* (Hara et al. 2003 – cited by Koseki et al. 2008), *Aspergillus nidulans* (Bauer et al. 2006 – cited by Koseki et al. 2008), and *Volvariella volvacea* (Ding et al. 2006 – cited by Koseki et al. 2008). Radford (2006) found eleven endoglucanases belonging to GH 61 family in *Neurospora crassa.*

8 Laccase

Lignin is the third major component of lignocellulose, forming a macromolecular complex that surrounds cellulose fibers (Saha 2003). After acid or enzymatic hydrolysis of polysaccharides, lignin is the H_2O-insoluble residue obtained. It is essential in structural support and in plant defense response. It also provides impermeability to the cell wall of plant vascular tissue, which is needed to water conduction (O'Malley et al. 1993).

For a long time it was believed that peroxidases were the only cell wall-associated oxidative enzymes involved in the polymerization step of lignin biosynthesis. After many years of disbelief it was revealed the participation of laccase, first described in 1883 but only later characterized, and laccase-like oxidases in lignification processes (Wallace and Fry 1999). However, Mayer and Staples (2002), reviewing later studies with fungi and plant laccases, exposed the possibility of fungal laccases not to take part in lignification processes. In plants these enzymes would be involved in cell-wall formation, but not exactly in lignification.

The presence of laccase, alone or together with lignin peroxidase and manganese peroxidase, has been detected in a wide variety of white rot fungi, making clear its fundamental role during delignification. The structure of many of these laccases have been described and, although the catalytic site seems to have been highly conserved and despite the fact that lignocellulose is a common substrate, the rest of the molecule appears to show very wide variability (Mayer and Staples 2002). The presence of phenolic compounds, which act as mediators, enhance the stability of some of them, increasing the lignocellulose breakdown ability (Eggert et al. 1996).

The phenolic substrates are also oxidized by laccases, which use oxygen as the electron acceptor. This enzyme is a blue metalloprotein, containing three copper ions that participate in a four-electron transfer to molecular oxygen, which is reduced to form water (Solomon and Lowery 1993). Laccase, which is glycosylated, belongs to the group of the multicopper enzymes along with ascorbic acid oxidase, ceruloplasmin and others, being able to oxidize a wide range of phenolic substrates (Messerschmidt and Huber 1990). Circular dichroism and electron paramagnetic resonance have already been used in the study of the metal centers of fungal laccases, showing that they are relatively stable compared to the secondary structure of the protein (Bonomo et al. 2001). Basically, any substrate with characteristics similar to *p*-diphenol will be oxidized by laccases. In addition, at least some of the fungal laccases can also oxidize monophenols such as cresol, and some are able to oxidize ascorbic acid (Mayer and Staples 2002).

Laccases are divided into two groups: the fungi one, having appeared in most of the studied organisms, and in the higher plants one, where they are less frequently found. The enzymes known as laccase-like enzymes are said to belong to bacteria and insects (Mayer and Staples 2002). Laccase has been shown to be an important virulence factor in many diseases caused by fungi, protecting them from the toxic phytoalexins and tannins in the host environment (Pezet et al. 1992). Its activity may also contribute to cell-wall reconstitution in regenerating protoplasts of higher

plants (De marco and Roubelakis-Angelakis 1997). Laccase-like oxidase activity can be localized in the differentiating xylem of both herbaceous and woody angiosperms (Bao et al. 1993).

9 Industrial Application of Enzymes

The majority of used industrial enzymes are hydrolytic, including proteases and GHs. The global market for industrial enzymes was estimated to totally $2 billion in 2004, and its annual growth rate predicted to be between 4 and 5% (Kirk et al. 2002; Turner et al. 2007). Given the natural abundance of holocellulosic materials, many microorganisms produce enzyme systems to hydrolyze holocellulose completely into simpler sugars that can be used as energy, feed and food sources. The enzymes described in this chapter are involved in the cleavage of plant cell wall structure. As the industrial processing of cell wall structure involves degradation of its complex components, it is obvious that enzyme systems capable of degrading those components can be applied in various industrial processes (Table 1).

The use of enzymes in the production of feed is an important sector of agrobusiness, with an annual world production exceeding 600 million tons and a turnover of >50 billion dollars. β–Glucanases, xylanases, mannanases, pectinases and feruloyl esterases are used in animal feed to reduce viscosity, leading to an increase on the absorption and release of nutrients through the hydrolysis of nonstarchy polyssacharides, such as β-glucans and arabinoxylans, and also increasing feed conversion rates and weight gain (Bhat 2000; Polizeli et al. 2005; Topakas et al. 2007; Uenojo and Pastore 2007; Moreira and Filho 2008). Morevover, the increase in digestibility is well correlated with the decrease in the degree of substitution of the hemicellulose polymers with arabinosyl residues, promoted by the addition of α-L-Arabinofuranosidases (Numan and Bhosle 2006).

Hemicellulolytic enzymes have shown potential in the pulp and paper industry for the modification of wood fibers. Mixtures of hemicellulases and cellulases have also been used to increase the drainage of secondary fiber during papermaking (Gerber et al. 1999). In the enzymatic treatment for pulp bleaching, β-mannanase and its accessory enzymes are able to cleave the mannan portion in pulps selectively without affecting cellulose (Filho 1998). Xylanases can also be industrially applied in the pulp biobleching, leading to a decrease in the amount of organochlorine compounds in bleach plant effluents and elemental chlorine – free (ECF) system (Medeiros et al. 2002). Feruloyl esterases, if used together with xylanase and other xylan-degrading enzymes, could partially disrupt and loosen the cell wall structure, enhancing the lignin extractability and reducing the chlorine consumption at the subsequent bleaching stages (Topakas et al. 2007). The application of α-L-Arabinofuranosidases can further enhance the delignification of pulps. The enzyme acts to release the arabinose side chain that retards the action of other bleaching enzymes (Numan and Bhosle 2006). It was also shown that laccases, manganese-dependent peroxidases, and lignin peroxidases, produced by an isolate of the fungus *Flavodon flavus,* were able

Table 1 A summary of the main biotechnology applications of some plant cell wall-degrading enzymes

Enzyme	Application	Sector
Cellulase	Decrease in the viscosity and maintenance of the texture of fruit juices	Juice industry
	Controlling coronary heart disease and artherosclerosis	Pharmaceutical industry
	Reducing food spoilage	Food industry
	Protein degradation	Detergent
Cellulase and hemicellulase	Lignocellulose breaking down	Biofuel
Xylanase	Improvement of nutrient uptake	Animal feed
	Paper bio-bleachin through lignin removal	Paper and pulp industry
	Separation and isolation of starch and gluten from wheat flour	Food industry
Endoglucanase	Separation and isolation of starch and gluten from wheat flour	Food industry
Pectinase	Decrease in the viscosity and maintenance of the texture of fruit juices	Juice industry
	Increased yields	Wine industry
	Reducing food spoilage	Food industry
	Nutrient uptake improvement	Animal feed
	Alteration of the sensory properties of fruits and vegetables	Food industry
	Paper bio-bleaching through lignin removal	Paper and pulp industry
	Increased yields	Oil extraction
	Remove mucilage coat from the coffee beans	Coffe processing
Pectin esterase	Improvement in the clarification of cider	Wine industry
	Production of high quality tomato ketchup and fruit pulps	Food industry
Rhamnogalacturonase	Production of cloud stable apple juice	Juice industry
Endoxylanase	Improvement in the texture and quality of bakery products	Food industry
β-Glucosidase	Improvement in the aroma of wines	Wine production
	Alteration of the sensory properties of fruits and vegetables	Food industry
Polygalacturonase	Improvement in pressing fruit mashes and high color extraction	Juice Industry
β-Mannanase	Food safety and preservation	Food Industry
	Nutrient uptake improvement	Animal Feed
	Paper prebleaching reducing loss of fiber yield	Paper and Pulp Industry
	Viscosity reduction by hydrolysis	Coffe processing
	Clarification of juices and wines	Juice and wine industry
	Increased yields	Oil extraction

(continued)

Table 1 (continued)

Enzyme	Application	Sector
β-Glucanase	Food safety and preservation	Food industry
	Improvement in primary fermentation, filtration and quality of beer	Beer brewing
	Nutrient uptake improvement	Animal feed
Endo-mannanase	Production of water-soluble dietary fibers to enrich the fiber content of foods	Food industry
Feruloyl esterase	Nutrient uptake improvement	Animal feed
α-L-Arabinofuranosidase	Paper bio-bleaching through lignin removal	Paper and pulp industry
	Decrease in the hemicellulose polymers substitution degree	Animal feed

to act in the decolorization of the effluent from a Kraft paper mill bleach plant, being potential candidates for bioremediation of colored industrial effluents (Mayer and Staples 2002).

Cellulases have achieved worldwide success in textile and laundry because of their ability to modify cellulosic fibers in a controlled and desired manner, so as to improve the quality of fabrics. Bio-stoning and bio-polishing are the best known current textile applications of cellulases, but they can also be used in household washing powders, since they enhance the detergent performance and allow the removal of small, fuzzy fibrils from fabric surfaces and improve the appearance and color brightness (Bhat 2000).

The wine making process requires the extraction of juice from grapes and subsequent fermentation of the juice by yeast. The addition of exogenous glucanases and pectinases are known to improve not only the wine quality, but also its overall production (Bhat 2000). One of the most important characteristics of wine quality is its aromatic fragrance. Terpenols are strongly aromatic molecules, and most of them are linked to disaccharide moieties, in which the major terminal nonreducing sugar is α-L-arabinofuranose that can be released by the action of α-L-Arabinofuranosidases (Numan and Bhosle 2006).

The beer brewing technology is based on the action of enzymes activated during malting and fermentation. Malting of barley depends on seed germination, which initiates the biosynthesis and activation of α- and β-amylases, carboxypeptidase and β-glucanase that hydrolyze the seed reserve. All these enzymes should act in synergy under optimal conditions to produce high quality malt (Bhat 2000).

Currently, a combination of pectinases (pectin lyase, pectin methylesterase, endo and exo-polygalacturonases, pectin acetylesterase, rhamnogalacturonase, endo and exo-arabinase), cellulases (endoglucanases, exoglucanases and cellobiases) and hemicellulases (endo and exo-xylanases, galactanases, xyloglucanases and mannanases) are used in the extraction and clarification of fruit and vegetable juices (Bhat 2000). The α-L-arabinofuranosidases are receiving attention for their applications in fruit juice clarification because they specifically remove the 1,3-side chains present on the main 1,5-linked arabinan chains. The resulting 1,5 arabinans in the precipitate are hydrolyzed by the α-1,5 arabinases, increasing their solubility (Numan and Bhosle 2006).

Enzyme-catalyzed conversion of sugarcane, sugar beet, corn or wheat to ethanol by distillers yeast *S. cerevisiae* is the current process for the industrial production of bioethanol (Sorensen et al. 2005). These substrates contain nonfermentable hemicelluloses, which accumulate during the process of ethanol production. Enzymatic hydrolysis can be used for the hydrolysis of arabinoxylans in hemicelluloses to monosaccharides. The complexity and heterogeneity of the arabinoxylans demand also complex enzyme systems that should depolymerize and cleave side groups to degrade hemicelluloses to pentoses monosaccharides (Numan and Bhosle 2006; Kumar et al. 2008). However, cellulose bioconversion into fermentable sugars by cellulases is a biorefining area that has invested enormous research efforts because of its crucial importance to bioenergy production, since cellulose is the most prevalent sugar in nature.

In the processing of instant coffee, where the extracts of coffee beans are concentrated by evaporation and dried by freeze drying, β-mannanase is responsible for the hydrolysis of galactomannan in coffee extracts, leading to a viscosity reduction (Moreira and Filho 2008).

10 Conclusions

The complexity of lignocellulosic substrates is reflected in the battery of main-chain- and side-chain-cleaving enzymes required for their hydrolysis. Such enzyme systems are not only of academic interest since they have potential applications in the bioconversion of agro-industrial residues. As the nature of plant cell wall polysaccharides is heterogeneous, a complex mixture of enzymes is required for their degradation. Efficient conversion of lignocellulosic material into useful products by enzymatic degradation, e.g., bioethanol production, requires a complex, but balanced mixture of enzymes, including hydrolytic and oxidative enzyme systems. The search for new renewable sources of energy has increased in the past 10 years. In this context bioethanol has shown up as a candidate for substituting the old fashioned fossil fuels. The increased demand for fermentable sugars caused the use of lignocelluloses from agricultural residues as substrates for microorganisms to work on. Fungus and bacteria's enzymes and complexes have being studied and their use could promote a sustainable way of dealing with these residues. The leftover from sugarcane, soy, corn, wheat, cotton what was thought to be worthless is now seen a possible gold mine, as well as a mean of saving the world from some of the human's wastes. The costs of the studies make the new technologies not very advantageous yet but there are some characteristics that could still be investigated and exploited.

The understanding of enzymatic synergism is a key for improving the cell wall degradation and sugar production. In many situations the dependence on others can be a better choice than being all by itself. Also, the promiscuous behavior of some enzymes is a desirable characteristic, for its wide range of suitable substrates. But when compared to mankind's situation, it is questionable whether the nature's push towards diversification is a step back when faced with the modern tendency of specialization.

Should enzymes be faithful and direct all their energy to one target or should they be promiscuous and share their performance between many? Anyway, apart from the enzymatic dilemma, the establishment of sugarcane and other cultures as energy source plants, especially in developing countries such as Brazil, could bring positive and negative effects, depending on the focus and in the way it will be treated from now on.

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Chapter 7
Enzymes in Bioenergy

Viviane I. Serpa and Igor Polikarpov

1 Introduction

Biomass is a renewable energy source which is a result of solar energy conversion during the process of photosynthesis. The chlorophyll in plants captures the solar energy and converts carbon dioxide and water into carbohydrates, which can, then, be transformed into other usable forms of energy like transportation fuels such as ethanol, for example.

Biomass is composed mainly of three biobased chemicals namely cellulose, hemicellulose, and lignin. Together, they are frequently designated as lignocellulose, a composite material of rigid cellulose fibers embedded in a cross-linked matrix of lignin and hemicellulose that bind the fibers. This structure of plants naturally resists to decomposition from microbes and enzymes and this collective resistance is known as "biomass recalcitrance."

The complex structure of biomass requires proper pretreatment to enable efficient saccharification of cellulose and hemicellulose components to their constituent sugars. The goal of a pretreatment is to alter or remove structural and compositional impediments to hydrolysis in order to improve the rate of enzyme hydrolysis and increase yields of fermentable sugars.

Breakthrough technologies are needed to overcome barriers to developing cost-effective processes for converting biomass to bioenergy, and the knowledge of the molecular mechanisms of the enzymatic hydrolysis of lignocellulosic feedstocks is one of the important parts of this process.

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2 Cellulose

Cellulose is the most important component of sugarcane bagasse available for bioconversion. It is also the most abundant renewable biosource produced in biosphere (~100 billion dry tons/year) and its systematic use for biofuels and biobased products is important for the sustainable development (Zhang and Lynd 2004). Cellulose is a linear polymer consisting of D-anhydroglucopyranose joined together by β-1,4-glycosidic bond with degree of polymerization (DP) from 100 to 20,000 (Osullivan 1997; Zhang and Lynd 2004; Zhang et al. 2006). The repeating unit of cellulose is anhydrocellobiose, which is strongly coupled to adjacent cellulose chains by hydrogen bonds and van der Waals interactions (Zhbankov 1992; Zhang and Lynd 2004). Crystalline regions of cellulose are intercalated with less ordered amorphous regions. The cellulose molecule is very stable, with a half-life of several million years for spontaneous β-glucosidic bond cleavage at room temperature, which implicates that practically all cellulose degradation in Nature is accomplished by enzymes action (Wolfenden and Snider 2001; Berner 2003; Zhang et al. 2006).

Cellulosic bioconversion is a complex process and requires the synergistic action of at least three major enzymatic components consisting of endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91), and β-glucosidases (3.2.1.21) (Henrissat 1994; Teeri 1997; Zhang and Lynd 2004; Zhang et al. 2006) (Fig. 1).

Exoglucanases progressively cleave cellulose chains at the reduced and nonreduced termini to release soluble cellobiose or glucose. Endoglucanases randomly hydrolase accessible intramolecular β-1,4-glucosidic bonds of cellulose chains to

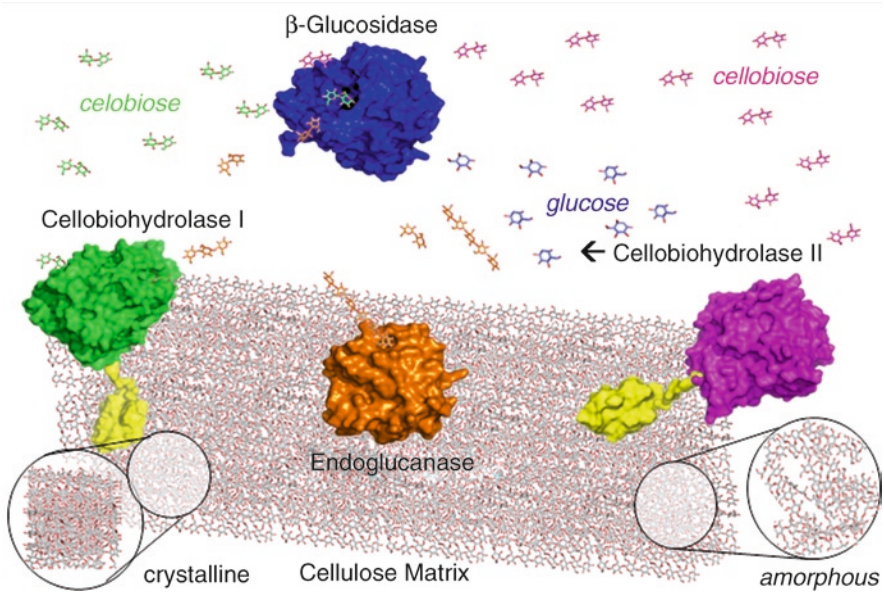

Fig. 1 Scheme showing the mechanisms of cellulase's enzyme action on the cellulose matrix

produce new termini available to exoglucanase attack. Hydrolysis is completed by β-glucosidases that hydrolyze cellobiose into glucose. β-glucosidases are generally responsible for the regulation of the whole cellulolytic process and its rate-limiting factor during enzymatic hydrolysis of cellulose, as both endoglucanase and exoglucanase activities are often susceptible to cellobiose inhibition, while β-glucosidases are inhibited by glucose (Harhangi et al. 2002; Kaur et al. 2007).

Commercial cellulose preparations supplementing with β-glucosidases have been reported to increase the rate of biomass hydrolysis and play an important role for cellulose saccharification on an industrial scale (Brumbauer et al. 1998; Kaur et al. 2007). In addition, in recent years, some β-glucosidases have received increased attention due to their ability to catalyze transglycosylation reactions that have great importance in food, cosmetic, and tobacco industries (Jerkovic and Mastelic 2004).

Elimination of cellobiose by β-glucosidases action relieves cellobiose inhibition of glucanases leading to a more efficient action of these enzymes mixtures on cellulose substrate. Thus, primary hydrolysis, driven by endo- and exoglucanases, occurs on the surface of solid substrates yielding soluble sugars with DP up to 6 into liquid phase. Secondary hydrolysis of cellobiose to glucose, catalyzed by the action of β-glucosidases, occurs in the liquid phase.

Synergism between endoglucanases and exoglucanases is the most widely studied type of synergy and is among the most quantitatively important for hydrolysis of crystalline cellulose. However, other types of synergism, for example, between endoglucanase and endoglucanase and intramolecular synergy between catalytic domain and cellulose-binding module (CBM), have also been proposed (Tuka et al. 1992; Walker et al. 1992; Din et al. 1994; Zhang and Lynd 2004).

Hydrolytic enzymes are also relatively expensive and difficult to produce in bulk quantities, and therefore, significant reduction of production costs will be important for their commercial use in the second generation of the bioethanol production. Although a number of microorganisms, including fungi and bacteria, have the capacity to degrade plant biomass, most commercially available enzymes are currently produced by genetically engineered strains of filamentous fungi *Trichoderma reesei* (*Hypocrea jecorina*) and *Aspergillus niger* (Kirk et al. 2002; Cherry and Fidantsef 2003).

Cost of the cellulolytic enzymes represents significant fraction of the second generation ethanol, therefore the hydrolytic activities of individual enzymes should be enhanced and the industrial cellulase cocktails must be balanced to guarantee improved efficiency of pretreated biomass conversion (Himmel et al. 1999; Cherry and Fidantsef 2003).

Glycoside hydrolases (GH, EC 3.2.1.-) are a widespread group of enzymes that hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a noncarbohydrate moiety. A widely used classification of GH, based on amino acid sequence similarities and hydrophobic cluster analysis (Henrissat 1991; Henrissat and Bairoch 1993), currently separates these enzymes in 112 GH families (GHF, http://www.cazy.org/). Cellulases and β-glucosidases are members of GH group of enzymes and their structural studies have provided

important insights into molecular mode of their catalytic function. Out of all fungi cellulases, *T. reesei* (*H. jecorina*) and *Humicola insolens* cellulases have been most intensively studied structurally.

Glucosyl hydrolases generally have a modular structure, which consists of a catalytic domain and one or more noncatalytic carbohydrate-binding modules joined by a highly glycosylated peptide linker. Deletion of the noncatalytic domains has no effect on the activity against soluble polysaccharides, but reduces activity against complex and insoluble substrates containing crystalline cellulose (Raghothama et al. 2000).

The major components of the cellulolytic enzyme system of *T. reesei* are well adapted for hydrolysis at the surface boundary of the solid substrate, and most enzymes have the two-domain organization with a large catalytic core domain (CCD) connected to a small cellulose-binding module (CBM) via long, heavily O-glycosylated linker peptide (Fig. 2).

The CBMs of CBH II (Cel6A) and endoglucanase II (Cel5A) are located at the N terminus, those of CBH I (Cel7A), endoglucanase I (Cel7B), and endoglucanase V (Cel45A) are at the C terminus, whereas endoglucanase III (Cel12A) does not possess CBM. The CBMs of all *T. reesei* cellulases show a remarkable homology with sequence identity of about 70% (Teeri et al. 1987). The structures of the CBMs from *T. reesei* CBH I (Cel7A, Kraulis et al. 1989; Mattinen et al. 1997) and EGI Cel7B (Mattinen et al. 1998) have been solved by the NMR technique. CBMs' interactions with cellulose were studied by the isothermal titration microcalorimetry

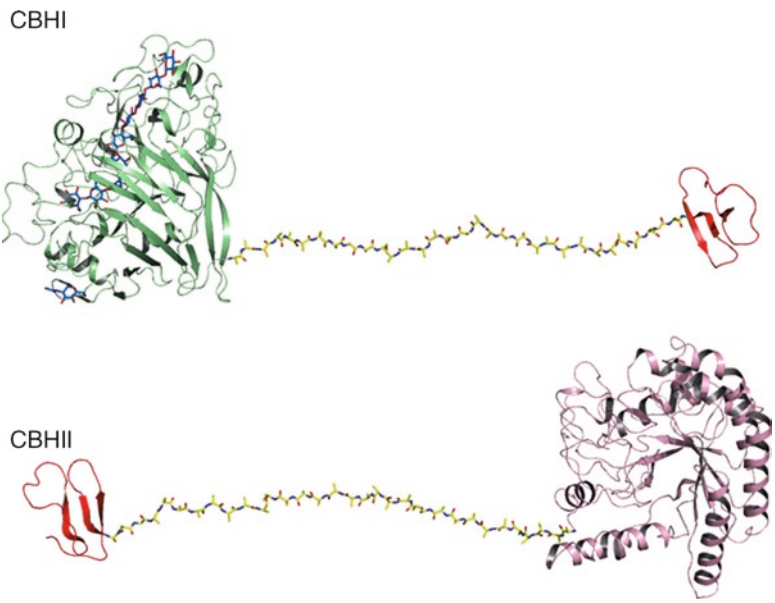

Fig. 2 3D models of CBHI and CBHII. Large catalytic core domain (CCD) (larger domains) is fused by a long linker to a small cellulose-binding module (CBM, smaller domains)

and were found entropically driven for some (Creagh et al. 1996; Sakka et al. 2003), but enthalpically driven for other CBMs (Boraston 2005). It has been proposed that the two domains, catalytic domain and CBM, of cellobiohydrolases interact simultaneously with the cellulose surface, each with different binding specificities, thereby enabling the enzyme to move along the cellulose surface through two-dimensional lateral diffusion (Stahlberg et al. 1991, 1996).

The major components of *T. reesei* cellulolytic machinery are exoglucanases CBHI and CBHII, which account for almost 80% of the total cellulolytic protein produced by the fungus. The CCD of CBHI (Cel7A) belongs to family 7 of the glycosyl hydrolases (Davies and Henrissat 1995) with a central structural motif which is a large, mainly antiparallel β-sandwich (Fig. 2) (Divne et al. 1994, Stahlberg et al. 1996). CBHI is a retaining exo-cellulase that hydrolyzes the β-1, 4-linkages of a cellulose chain from its reducing end (Knowles et al. 1988; Claeyssens et al. 1990; Divne et al. 1994); whereas CBHII (Fig. 2) cleaves β-glucosidic bonds by inverting mechanism and mainly from the nonreducing end (Davies and Henrissat 1995). Its CCD, from a GHF 6, folds into an unusual parallel seven-stranded α/β barrel (Rouvinen et al. 1990). The catalytic domain structures of CBHI and CBHII both reveal tunnel-shaped structures formed by loops (Fig. 3).

A striking feature of the CBHI structure is an extensive 50 Å long cellulose-binding tunnel formed by four surface loops, which runs along the concave face of the sandwich and contains the active site at its far end (Figs. 1 and 2). The polysaccharide chain slides through the substrate-binding tunnel, and every second glycosidic bond is correctly presented to the catalytic apparatus, explaining why these enzymes

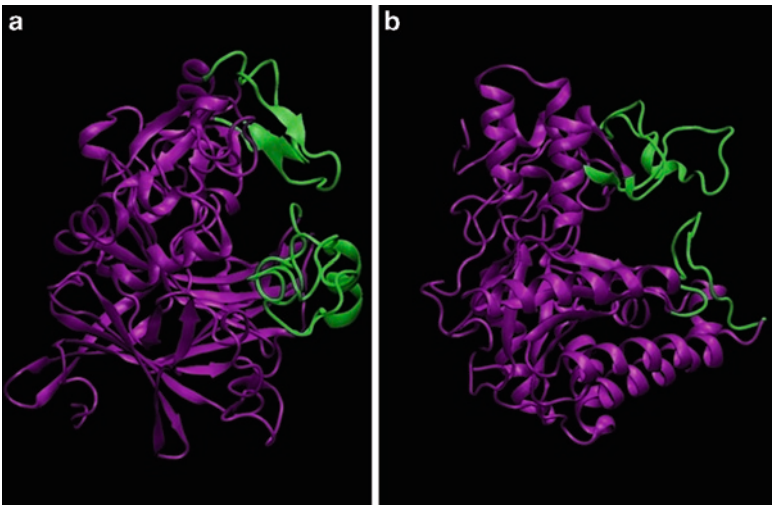

Fig. 3 CCDs of *T. reesei* CBH I (Cel7A) (**a**) and CBH II (Cel6A) (**b**). Loops highlight tunnel roofs. Tunnel lengths are 50 and 20 Å long (out of the plane of the paper) in CBH I (Cel7A) and CBH II (Cel6A), respectively

progressively liberate disaccharide units (cellobiose) from the cellulose chain (Divne et al. 1994).

The roles of the catalytic residues (Glu212, Asp214, and Glu217) of CBHI from *T. reesei* have been investigated by site-directed mutagenesis, enzymatic, and X-ray crystallography studies (Stahlberg et al. 1996). Mutation of any of these amino acid residues significantly impaired the catalytic activity of the enzyme. Site-directed mutagenesis of Cel7A (CBHI) was also used to engineer the enzyme to more alkaline optimum. E223S/A224H/L225V/T226A/D262G Cel7A mutant, constructed based on the sequence comparison with Cel7B (endoglucanase I) of *H. insolens* presented increase in pH optimum combined with the relief of its inhibition by cellobiose. In the catalytic domain of CBHII (Cel6A), two well-ordered loops form an active site tunnel which is 20 Å long (Rouvinen et al. 1990), also allowing for a structural interpretation of the processive action of this exoglucanase.

CBHI and CBHII are very potent enzymes, which can achieve complete, although slow, hydrolysis of crystalline cellulose, gradually decreasing the DP of cellulose, even without the help of endoglucanases (Teeri 1997). This is a slow process, since the exoglucanases have to associate with the insoluble cellulose, destruct its tight packing, and guide a single cellulose chain into the active site tunnel. Recent findings from a related chitinase research suggested that processivity, beneficial for hydrolysis of insoluble substrates, could in fact be a drawback for hydrolysis of soluble substrates (Eijsink et al. 2008). Furthermore, decrease in processivity of cellulases seems to be positively correlated with the improvement of enzyme activity toward soluble substrates (von Ossowski et al. 2003; Varrot et al. 2003; Li et al. 2007).

A recent study, combining X-ray crystallography, site-directed mutagenesis experiments, and molecular dynamics simulations, shed more light on the fine details of a catalytic mechanism of CBHII (Cel6A) and allowed to confirm the role of residue D221 as the catalytic acid (Koivula et al. 2002). D175 is shown to affect protonation of D221 and to contribute to the electrostatic stabilization of the partial positive charge in the transition state (Koivula et al. 2002). Previous mutagenesis studies already shown that mutation Y169F led to a significant increase of association constants of the mutant enzyme for cellobiose and cellotriose with simultaneous decrease of constants towards cellotriose and cellotetraose (Koivula et al. 1996). In addition, a change in the pH activity profile was observed. These data suggest that Y169, on interacting with a glucose ring entering the second subsite in a narrow tunnel, helps to distort the glucose ring into a more reactive conformation and also indicates that Y169 affects the protonation state of the active site carboxylates, D175 and D221 (Koivula et al. 1996).

The structural basis for ligand binding and CBHII processivity has been challenged by the structure of cellobiohydrolase Cel6A from *H. insolens* (Varrot et al. 1999, 2003). This crystal structure, highly homologous to *T. reesei* CBHII, reveals that in fact both of these macromolecules should allow for some degree of endo-cellulase activity, presumably mediated by the flexibility of its active site loops (Varrot et al. 1999).

The structure of the *T. reesei* endoglucanase Cel7B (EGI) catalytic domain, homologous to Cel7A (CBHI), revealed that the loops forming the tunnel in CBH I are absent in EG I, and instead of a tunnel, EG I has a more open substrate-binding

cleft (Kleywegt et al. 1997). The same general features can be predicted from the sequence comparisons of other endoglucanase–exoglucanase pairs, suggesting that their modes of action on polymeric substrates are dictated by the shape of their active sites. Endoglucanases with open active sites can bind to and act in the middle of the glucan chains, while exoglucanases with tunnel-shaped active sites are mostly, but not exclusively, confined to the cellulose chain termini. It was shown that deletion of one of the surface loops that covers the active site of CbhA, a family 6 cellobiohydrolase, does enhance the "endolytic" characteristics of this exo-enzyme (Meinke et al. 1995). This discovery finds its support in a crystal structure of endoglucanase Cel6B from *H. insolens* (Davies et al. 2000), which is related to its cellobiohydrolase counterparts by reduction in just a single surface loop, i.e., represents a naturally occurring version of the cellobiohydrolase mutant constructed by Meinke et al. (1995).

Shortening of the cellulose-enclosing loop of *T. reesei* cellobiohydrolase CBHI (Cel7A) by deleting eight residues from the tip of the loop increased both k_{cat} and K_M and produced reduced product inhibition, increased activity on amorphous cellulose, and decreased by half the original activity remained on crystalline cellulose (von Ossowski et al. 2003). Stabilization of the exo-loop by the disulphide bridge enhanced the activity on both amorphous and crystalline cellulose. The processive action of the enzyme was highest with *T. reesei* Cel7A wild-type enzyme and smallest with the deletion mutant on both substrates. However, since enzymes acting on lignocellulosic substrates often exhibit subtle functional differences reflecting details of their substrate specificity, the conclusions drawn for one enzyme in a given family do not necessarily apply to another. Thus, *Phanerochaete chrysosporium* Cel7D is able to maintain high processivity, leading to efficient action on crystalline cellulose, in spite of its shorter active site loops and weaker binding in the product subsites +1/+2 (von Ossowski et al. 2003). These natural differences, and the fact that extensive alterations of the exo-loop can be made without disturbing the structural and catalytic integrity of the enzyme, suggest that the balance between product inhibition, nonproductive binding, catalytic rate, and progressivity can be further optimized by protein engineering for enhanced performance on different cellulosic substrates.

Crystal structure of *T. reesei* endoglucanase III (Cel12A), a small 24.5 kDa enzyme, which belongs to glycoside hydrolase family (GHF) 12 cellulase and lacks a CBM, reveals that its fold is similar to GHF11 and the active site resembles that of GHFs 7 and 16 (Sandgren et al. 2001). Its complexes with cellobiose, cellotetraose, cellopentose, and a thio-linked cellotetraose derivative have been determined and were used to map the noncovalent interactions between the enzyme and the glucosyl chain bound in subsites −4 to +2 of the enzyme, thus revealing structural features of the mechanism and function of GH 12 cellulases (Sandgren et al. 2004). Rational mutation of *T. reesei* Cel12A based on the amino acid and 3D structure comparison with related enzyme from thermostable *Humicola grisea* resulted in considerable increase in its thermostability (Sandgren et al. 2003a). *T. reesei* Cel12A P201C mutant showed the increase in T_m of 3.9°C as compared with the wild-type enzyme, whereas correspondent mutations of free cysteine residues in *H. grisea* led to a less stable enzyme (Sandgren et al. 2003a). A similar strategy, based on the sequence comparison with *Hypocrea schweinitzii* and *Streptomyces*

sp.11AG8, rendered an active *T. reesei* Cel12A A35V mutant with T_m 7.7°C higher than wild type (Sandgren et al. 2003b).

Despite a wealth of structural information on exo- and endoglucanases from *T. reesei* obtained to date, there are a number of questions still to be solved. Notably, no structural information is available for several *T. reesei* endoglucanases including endoglucanase II. EGII (Cel5A), a member of GHF 5, is responsible for most of the endoglucanase activity produced by *T. reesei*. Lack of Cel5A production reduces the endoglucanase activity in the culture supernatant, produced by this fungus, by as much as 55% (Suominen et al. 1993; Qin et al. 2008). To be able to comprehend fine mechanistic details of Cel5A function, it would be important to determine its 3D structure and to obtain information about the enzyme dynamics and mobility.

The wealth of information provided by the GH structure analysis demonstrates that the substrate specificity and the mode of action of these enzymes are governed by fine and particular details of their three-dimensional structures rather than by their global architecture (Davies and Henrissat 1995). Improvement of sampling in structure–functional space for cellulases and glucoside hydrolases in general is of a crucial importance for rational protein engineering of exo- and endoglucanases, which depends on the availability of 3D structure of the enzyme and homologous proteins and relies on our understanding of their structure, dynamics, and structural elements which determine the enzymatic action techniques (Zhang et al. 2006).

At present, only limited structural high-resolution structural information is available for entire two-domain GH, mostly obtained for enzymes with short linker peptides (Fujimoto et al. 2000; Pell et al. 2004). Even then, the lack of electron density for the linker residues indicated disorder of the linker. At present, no high-resolution structure of a single intact multidomain cellulase, consisting of the catalytic core, linker, and CBM, has been determined, most probably hampered by the intrinsic flexibility and disorder of their long and extended linker peptides. Since in Nature CBM and CCD cellulase domains function in a concerted way, full-length structures of cellulases are important for our comprehension of their function. To overcome the problem, low-resolution structural analysis in solution using small-angle X-ray scattering or electron microscopy might be useful in providing important insights into cellulase tertiary and quaternary organization.

Pioneering studies of intact *T. reesei* CBHI and CBHII by SAXS, which have been conducted at the end of 1980s, provided important insight into the tertiary organization of these two cellulase domains (Abuja et al. 1988a, b). The extended "tadpole" architecture of the exoglucanases has been described, but these early results were imperfect because of the lack of known three-dimensional structure for the isolated cellulase domains, as well as the limitations of equipment and software at that time. Recently, modern SAXS studies of *H. insolens* cellulase Cel45 and chimera fusion enzyme Cel6A–Cel6B using synchrotron radiation have been done, revealing important, and much more precise, information on the tertiary structure of the enzymes and the conformational flexibility of the connecting linker region (Receveur et al. 2002; von Ossowski et al. 2005).

3 Hemicellulose

Xylan is the second most abundant polysaccharide in Nature (Beg et al. 2001) and is the principal type of hemicelluloses, which is a linear polymer of β-D-xylopyranosyl units linked by (1–4) glycosidic bonds. In nature, the polysaccharide backbone may be added to 4-O-methyl-α-D-glucuronopyranosyl units, acetyl groups, α-L-arabinofuranosyl, etc. in variable proportions. Complexes of enzymes are responsible for the hydrolysis of xylan, but the main enzymes involved are endo-1,4-β-xylanase and β-xylosidase. There are two main types of xylanase activities: (a) endo-xylanases (endo-1,4-xylanases; EC 3.2.1.8), which show a preference for internal xylan bonds, and (b) exo-xylanases (exo-1,4-β-xylanases; EC 3.2.1.37), which demonstrate a preference for groups at the termini of xylan chains. These enzymes are produced by fungi, bacteria, yeast, marine algae, protozoans, snails, crustaceans, insect, seeds, etc., with the principal commercial source being filamentous fungi (Polizeli et al. 2005). In particular, filamentous fungi have demonstrated a great capability for secreting a wide range of xylanases, and those belonging to the genus *Aspergillus* and *Trichoderma* have been most extensively studied and reviewed among the xylan-producing fungi. In recent years, significant amount of information about the production of xylanases and fungi genetics has also been accumulated for the *Penicillium* species (Gordillo et al. 2006). Hydrolysis of β-glycosidic linkages is sponsored by a general acid catalytic reaction common to all glycanases, whereas substrate recognition is specified by subsites that interact with adjacent glycosyl units.

According to the classification of The Carbohydrate-Active Enzymes Database (http://wwww.cazy.org/) (Henrissat 1991; Henrissat and Bairoch 1996; Cantarel et al. 2009), the main enzymes involved in the hemicellulose degradation are grouped in several glycosyl hydrolase families (GH): endo-1,4-β-xylanase (EC: 3.2.1.8) GH 5, 8, 10, 11, 43; β-xylosidase (EC: 3.2.1.37) GH 3, 30, 31, 39, 43, 52 e 54; and α-L-arabinofuranosidase (EC: 3.2.1.55) GH 3, 43, 51, 54 E 62.

Xylanases are usually found in glycosyl hydrolase families 10 and 11. The xylanases from family 10 have a molecular mass of approximately 35 kDa and possess the TIM-barrel $(\beta/\alpha)_8$ topology (Davies and Henrissat 1995); while xylanases from the family 11 have a mass of approximately 22 kDa and a jelly-roll topology consisting of two twisted β-sheets stacked face to face. The structure has been likened to a right hand, with a two-β-strand "thumb" forming a lid over the active site with the active site located in the "palm" (Torronen et al. 1994; Oakley et al. 2003). GH10 xylanases have chameleon-like characteristics, with variable surface features facilitating their adaptations to different situations (Manikandan et al. 2006).

Structural and biochemical evidence suggests that xylanases mostly act by a double displacement manner, which results in retaining the anomeric configuration of substrate. However, some of the enzymes catalyze single displacement reactions with inversion of substrate configuration (Kulkarni et al. 1999; Rao and Mishra 1989).

The modular structures of xylanases are similar to other glycosyl hydrolysis. However, apart from the xylanases that contain domains that bind specifically to

xylan (xylan-binding modules or XBMs), there are several xylanases which contain domains that bind specifically to cellulose rather than xylan. The reason for this is probably the different complexity of the structures of xylan and cellulose. The cellulose is a uniform, crystalline, and ubiquitous ligand, whereas xylan can be very variable depending on the species or differentiation state of the plant. Most of these domains bind to crystalline cellulose and have a common structure consisting of a β-sheet core, with two or, more usually, three aromatic residues across one face. In the case of the XBM2b, from *Pseudomonas*, the aromatic rings form a twisted binding site, suitably oriented for interaction with the helical structure of xylan (Raghothama et al. 2000). Industrial demands for enzymes that can operate under extreme process conditions required several studies of extremophilic xylanases, in particular those from thermophiles, alkaliphiles, acidiphiles, and psychrophile xylanases. For instance, the understanding of the structural basis for protein thermostability is of considerable biological and biotechnological importance as exemplified by the industrial use of xylanases at elevated temperatures in the paper pulp and animal feed sectors. The reasons for the thermostability of the *Thermomyces lanuginosus* xylanase were analyzed by comparing its crystal structure with known structures of mesophilic family 11 xylanases. It appears that the thermostability is due to the presence of an extra disulfide bridge, as well as to an increase in the density of charged residues throughout the protein (Gruber et al. 1998).

Another study of the thermophilic xylanase from *T. lanuginosus* indicates that the enzyme consists of two β-sheets and one α-helix and forms a rigid complex with the three central sugars of xyloheptaose, whereas the peripheral sugars might assume different configurations thereby allowing branched xylan chains to be accepted (Singh et al. 2003).

The xylanases from alkalophilic organisms show different characteristics related to its structure. Comparison of the structure of BSX, from an alkalophilic *Bacillus* sp., with those of other thermostable and alkalophilic GH10 xylanases optimally active at acidic or close to neutral pH showed that the solvent-exposed acidic amino acids, Asp and Glu, are markedly enhanced in BSX, while solvent-exposed Asn was noticeably depleted. Then, a protein surface rich in acidic residues may be an important feature common to all alkali thermostable enzymes (Manikandan et al. 2006).

A conserved triad (Val-Val-Xaa, where Xaa is Asn or Asp) is a characteristic identified only in the xylanases from alkaliphilic organisms. The results implicate for the first time the concept of convergent evolution for XynII and provide a basis for research in evolutionary relationship among the xylanases from alkaliphilic and neutrophilic organisms (Rao and Mishra 1989).

A psychrophilic xylanase, which belongs to family 8, was isolated from Antarctic bacterium *Pseudoalteromonas haloplanktis* and studied by NMR analysis (Van Petegem et al. 2003). It has a different fold from that of other known xylanases and can be described as an $(\alpha/\alpha)_6$ barrel. Various parameters that may explain the cold-adapted properties were examined and indicated that the protein has a reduced number of salt bridges and an increased exposure of hydrophobic residues (Van Petegem et al. 2003).

The xylanases are inhibited by macromolecules (XIs) classified in three classes: *Triticum aestivum* xylanase inhibitor (TAXI) (Sansen et al. 2004), xylanase-inhibiting protein (XIP), and thaumatin-like xylanase inhibitor (TLXI) (Fierens et al. 2009). These proteins have been linked to plant defense like is in wheat, which contains high levels of the three classes of inhibitors (Vardakou et al. 2008).

The xylanases enzymatic hydrolysis of glycosides has been extensively studied. The reaction involves the formation and subsequent breakdown of a covalent glycosyl-enzyme intermediate via oxocarbenium-ion-like transition states. Structural analysis of enzymatic mechanism is possible because the covalent intermediate can be trapped on-enzyme using P-fluoro-substituted glycosides, which provide details of the intermediate conformation and noncovalent interactions between enzyme and oligosaccharide (Sabini et al. 1999).

The development of new analytical techniques and the commercial availability of new substrates have led to the purification and characterization of a large number of xylan-degrading enzymes. Furthermore, the introduction of recombinant DNA technology has resulted in the selection of xylanolytic enzymes that are more suitable for industrial applications (Sunna and Antranikian 1997). However, growing biotechnological interest in these enzymes demands their further biochemical, enzymatic, and structural studies.

4 Lignin

Lignin is a heterogeneous hydrophobic phenolic polymer and one of its main physiological functions is to "cement" the cellulose fibers in plants. It is the second most abundant natural raw material, being an integral part of lignocellulosic materials with the relative amount present varying over the approximate range of 10–30%. Lignin is formed from three primary precursors: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. Lignins may be divided into three broad classes, namely softwood, hardwood, and grass lignin, according to their composition in structural units (Carrott and Carrott 2007).

Lignin is degraded by different classes of enzymes, which are produced by different microorganisms, such as white-rot fungi for example, in several combinations: there are fungi producing lignin peroxidase (LiP) and manganese peroxidase (MnP), fungi producing MnP and laccase, fungi producing LiP and laccase, and fungi which produce neither LiP nor MnP, but laccase and aryl alcohol oxidase (AAO) or some other enzyme (Hatakka 1994). The best studied extracellular enzymes of white-rot fungi are lignin peroxidases (LiPs), manganese peroxidases (MnPs), and laccase. However, lignin degradation is a complex process and the enzymes have probably synergistic effects on each other (Tuomela et al. 2000).

The interest in these enzymes increased during past years due to their industrial utility for the degradation of the biomass lignin. The later process increases the accessibility of the cellulose and hemicelluloses, which is important for the production of fuels and chemicals from lignocellulosic biomass (Gottschalk et al. 2008).

P. chrysosporium, a white-rot fungus, is a potential producer of lignin peroxidases (LiPs) and manganese peroxidases (MnPs) and it makes this fungus a model organism of lignin-degrading enzymes production (Singh and Chen 2008; Wang et al. 2008).

Laccase (EC: 1.10.3.2), which belongs to the family of blue multicopper oxidases, is an enzyme secreted into the medium by mycelia of *Basidiomycetes*, *Ascomycetes*, and *Deuteromycete*; however, the highest amounts of laccase are produced by white-rot fungi (wood-decaying basidiomycetes). Laccase is an enzyme which oxidizes a variety of aromatic hydrogen donors. It catalyzes the one-electron oxidation of four reducing-substrate molecules concomitant with the four-electron reduction of molecular oxygen to water (Piontek et al. 2002).

The crystal structures of laccase have been solved for both active (full complement of coppers) and inactive (type-2 copper-depleted) forms for laccases from *Trametes versicolor* (Piontek et al. 2002) and *Coprinus cinereus* (Ducros et al. 1998), respectively.

The crystal structure of the laccase from *T. versicolor* allowed the identification of several amino acid residues that make hydrophobic interactions with the aromatic ring of the ligand and two charged or polar residues that interact with its amino group. One of them is a histidine that also coordinates the copper that functions as the primary electron acceptor and the second is an aspartate conserved among fungal laccases (Bertrand et al. 2002).

Another important enzyme involved in lignin degradation is a lignin peroxidase (EC 1.11.1.14), which oxidizes aromatic compounds by single electron abstraction. Crystallographic structure of lignin peroxidase from the white-rot fungus *P. chrysosporium*, for example, contains 343 amino acid residues, the heme, four carbohydrates, and two calcium ions. This lignin peroxidase shows the typical peroxidase fold and the heme has a close environment as found in other peroxidases (Choinowski et al. 1999). Moreover, experiments with cationic peroxidase from poplar indicated that Tyr residues on the protein surface are considered to be important for the oxidation activities with a wide range of substrates and potentially unique oxidation sites for the plant peroxidase family (Sasaki et al. 2008).

Manganese peroxidase (MnP – EC: 1.11.1.13) is an Mn-binding enzyme, which is unique in its ability to bind and oxidize Mn-II and efficiently release Mn-III. It is a heme-containing enzyme produced by white-rot fungi and is also part of the lignin-degrading system (Youngs et al. 2001).

A 1.45 Å resolution crystal structure of MnP complexed with Mn(II) provides an accurate view of the Mn-binding site (Sundaramoorthy et al. 2005). Sundaramoorthy et al. identified a heme propionate and the side chains of Glu35, Glu39, and Asp179 as Mn(II) ligands (Sundaramoorthy et al. 2005). Glycosylation at Ser336 and partial protonation of Glu39 in the Mn-binding site were also revealed by the structure (Sundaramoorthy et al. 2005). Binding of MnII or CdII, which is a reversible competitive inhibitor of MnII in manganese peroxidases, to the Mn-binding site affords considerable thermal stability to manganese peroxidase (Youngs et al. 2000).

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Chapter 8
Hydrolases from Microorganisms used for Degradation of Plant Cell Wall and Bioenergy

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1 Introduction

The first consideration one should be aware of regarding biomass is what it exactly means. The definition of biomass has received different meanings during the course of the years, since it was first used in the 1930s. Currently, it differs according to the purpose of its use. Biologically and etymologically, biomass encompasses everything which is alive on Earth. A broader definition would include the three domains of life – Archaea, Eukarya, and Bacteria – they being alive or dead, along with their wastes. However, some other definitions exclude water, considering biomass as being the dry weight of living beings and their wastes; others claim biomass means the biodegradable fraction of products, waste and residues from agriculture (including vegetal and animal substances), aquaculture, forestry and related industries, the biodegradable fraction of industrial and municipal waste as well as waste water sludge. Lastly, biomass can also be defined as mass provided by living and/or dead plants only. From an energetic approach, biomass is every renewable resource from organic matter which can be used to produce energy. Consequently, all these different ways to define biomass can lead to multi-interpretations of single information. Additionally, we could incur in the mistake of calculating the total biomass of a unit or place without any life in it. To avoid such misunderstanding, we propose and use herein the following terms:

- BIOMASS as being all three domains of life while alive.
- BIODMASS (pronounced bio-d-mass) being biomass plus their wastes and dead organisms.
- BIOEMASS (pronounced bio-e-mass) as the biomass used to produce energy being it any fraction of the original biodmass.

Everything in the universe can be summarized in atoms and energy. Although natural energy takes place daily in our cosmos regardless of any active actions of living beings, life on earth, especially for mankind and as it is known today, depends on

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energy drivingly and promptly produced. The demand for energy by transport and service sectors has increased greatly. Road transport has contributed the most to the increase in overall transport energy consumption (Shafiee and Topal 2009). According to International Energy Agency, between 1990 and 2005, road transport energy use increased by 41%. The possible dark side of this lies in the consequences of this increase, which brought CO_2 emissions up approximately 25%.

Despite theories of lack in relatedness between the amount of CO_2 and climate change, especially global warming (http://www.creationworldview.org/articles _ view.asp?id=67), there seems to be an agreement among the scientific community that less CO_2 released into the atmosphere would strongly favor the environment.

The world's most used energy sources, mainly for transport, are fossil fuels which presumably take millions of years to form (for a different interpretation see http://www.creationworldview.org/articles_view.asp?id=51). If the world continues to consume fossil fuels at 2006 rates, the reserves of oil, coal, and gas will last approximately an additional 40, 200, and 70 years, respectively (Shafiee and Topal 2009), but projections from the EIA show that the consumption tends to increase. Additionally, according to the same department (http://www.eia.doe.gov/emeu/steo/pub/ contents. html#GlobalCrude_Oil And_Liquid_Fuels), despite the stability on the demand for fossil fuels in 2008 and most of 2009, the economic recovery in the United States and elsewhere will lead to a rebound in oil demand growth perhaps yet this year. For some, this can be looked upon as a time gift from "Mother Nature" to humankind so a secure, reliable, and affordable energy source is found to replace the fossil ones.

Exploiting fossil fuel reserves to their full technical potential might bring undesirable, and possibly, irreversible results to the environment (Nel and Copper 2009). Currently, there are alternative energy sources being evaluated and studied. Converting bioemass into energy is one alternative, if not the one which will most achieve its goals. Other possible sources of energy are: geothermal, hidropower, nuclear, ocean, solar, and wind sources, among others.

2 Biomass and Biofuels

Human domination on Earth has changed its landscape greatly. Although when we think of bioemass, we primarily picture plants (wood), the total biodmass on Earth goes much beyond accounting for over two trillion tons of raw matter (Ramage and Scurlock 1996). A great amount of this is suitable to be used to produce energy by being burned and/or transformed into biofuels among others. Biofuel refers to any solid, liquid, or gas fuel that has been derived from bioemass. It can be produced from any carbon source that is easy to replenish when compared with fossil fuels.

There are many advantages to biofuels. The goal of any biofuel, regardless of the type, is for it to be carbon neutral and, therefore, reduce greenhouse gas emissions. Although the energy spent through crop production, transportation, and processing to generate biofuels plus the consumption of carbon through this route result in a balance different from neutrality, biofuels still prove to be substantially more environmentally friendly than their alternatives. Additionally, their production also helps in the development of

rural areas, ensure long-term fuel availability, and improve the economy equilibrium of many countries making them less dependent on imported products.

Biofuels can be obtained directly by extraction from nature, however, raw matter sometimes needs to be processed. The result can be gaseous, liquid, or solid forms of fuels, the latter form being in use ever since man discovered fire. Solid biofuels such as wood, grass cuttings, domestic refuse, charcoal, dried manure, and residues among others can be burned to produce energy. The most common gaseous biofuels are biogas and syngas. Liquid biofuels such as vegetable oil, ethanol, and biodiesel can be easily pumped and handled. This is the main reason why almost all vehicles use liquid form of fuel for combustion purposes.

3 Sugarcane and Bioemass

In this scenario, sugarcane, among many raw matters suitable to be used as bioemass, has increasingly drawn our attention. Brazil was colonized to a large extent to produce the sugarcane plant and extract sugar from it. After the legislation of 1931 requiring the addition of ethanol to gasoline, the production plants in Brazil increased their production (www.iea.org). very efficient and advanced.

1. This increase in ethanol demand associated to many research results in different fields such as genetic engineering, enzymes enhancement, mechanical improvements for the machinery involved, chemical products easing the crops among others, leaded to a more efficient and advanced process which included the growth, harvesting, and processing technology regarding sugarcane and the production of alcohol.
2. Besides the energy involved in the production of sugarcane, ethanol production needs very little external sources of energy due to its bagasse, which supplies almost all necessary energy for the industrial phase. The alcohol from sugarcane has a superior energy balance being able to result in eight times more fuel in the end of the production process than the amount of fossil fuel used to enable it.

Sugarcane crops are very stable when compared with corn and other plants. Furthermore, ethanol is biodegradable, presents a low toxicity to aquatic and soil forms of life, has virtually no sulfur and particulate emissions, reduces CO emissions and other toxic substances, can be blended with other fuels, is a source of hydrogen (fuel cells), has increased the market of flex fuel vehicles and now motorcycles (www.iea.org), it is an alternative fuel for boats and airplanes, and scientists are even willing to make plastic from it.

Brazil's sugarcane plantations are spread across almost every state, but being concentrated mostly in the central south and northeast. The biggest production comes from the São Paulo state with approximately 296 million tons of sugarcane being grown in 2007–2008 (UNICA). Brazil's production of sugarcane was around 493 million tons, and with the increase in the available area to be harvested in 2008–2009, the total sugarcane production had already increased to a half billion tons by January 2008 in only the central south region. Consequently, the production of ethanol has increased from 22.5 to 27 billion liters in 2008–2009 (http://brasilatual.com.br/sistema/index.php?s=etanol+2009). However, there is a high potential for extension

of crops in São Paulo and other states as well. At least 90 million hectares can still be used to plant sugarcane in Brazil (http://www.embrapa.br/imprensa/artigos/2008/A%20expansao%20da%20cana-de-acucar%20e%20a%20sua%20sustentabilidade.pdf). This fact makes Brazil a target place for national and international financial investments in research toward sugarcane improvement and alcohol production.

The discussion regarding the pros and cons of sugarcane crop expansion has become as intense as useless. It is obvious the country has a great potential for expansion without harming the Amazon forest, pantanal, or any other protected place, leaving enough space for cattle and food crops. However, we know sugarcane crops can be expanded only to a certain extent worldwide and that leaves us the only alternative of researching ways to increase productivity without increasing cropping areas.

4 Sugarcane Bagasse and Energy Production

Bagasse sugar and ethanol are mainly derived from sugarcane juice. Bagasse accounts for approximately 1/4 of the total sugarcane produced and it is usually burned to be used to produce energy for the production plant or sold to the animal food industry (http://www.agronline.com.br/agronoticias/noticia.php?id=2860) among other small usages for it. The straw is almost entirely left in the fields to be naturally recycled and part of the nutrients go back into the soil.

The straw corresponds to approximately 1/5 of the sugarcane production (UNICA). The total volume of bagasse will differ from production plant to production plant, as they use different machinery, some produce electricity and some do not, and whether or not the production plant sells energy. Overall, we can say that independently of the differences there might be among plants and amount of bagasse produced, if Brazil produces more than 500 million tons of sugarcane, there will be at least 125 million tons of bagasse and 100 million tons of straw. If we could efficiently use only half of the amount of bagasse (31.25 million tons of dry bagasse), there would be an increase of maybe more than 9 billion liters of ethanol production. Furthermore, an efficient methodology which brought affordability of extracting ethanol from bagasse from sugarcane would also provide insights to general methods of extracting ethanol from any wood.

5 Component of Sugarcane Bagasse

The sugarcane bagasse fibers contain approximately 46% cellulose, 25% hemicelluloses, and 21% lignin as the main components. The conversion of cellulose and hemicellulose fractions into ethanol by microbial enzymes should be considered a viable process. In order to evaluate the participation of enzymatic hydrolysis on sugarcane bagasse, a brief description of cellulose, hemicellulose, and lignin molecules is described below.

Cellulose is the main renewable carbon source in nature. It is a linear polysaccharide constituted by glucose subunits linked by β-1,4 bounds. Each residue is rotated 180° compared with its neighbors. The degree of polymerization (*DP*) of native cellulose is in the range of between 7,000 and 15,000, where:

DP = Molecular weight of cellulose / Molecular weight of one glucose unit.

The cellulose chains are oriented in parallel and form highly organized crystalline domains interspersed by more disorganized, amorphous regions (Fig. 1). Cellulose chains form numerous intra- and intermolecular hydrogen bonds, which account for the formation of rigid, insoluble microfibrils. The native crystalline form has a structure designated as type 1, which can be converted into type II by alkaline treatment. The two types differ in their intrachain hydrogen bonding pattern. Additionally, most native celluloses are composed of two slightly different forms. They are identified by solid state Nuclear Magnetic Ressonance spectrum in type I cellulose, denominated I_{α} and I_{β} which differ in their intermolecular hydrogen bonding and in proportions, depending on the cellulose source (Rahi et al. 2009). The binding of cellulose microfibrils to the hemicellulose xylan results in a noncovalently cross-linked cellulose-hemicellulose network which gives the wall tensile strength.

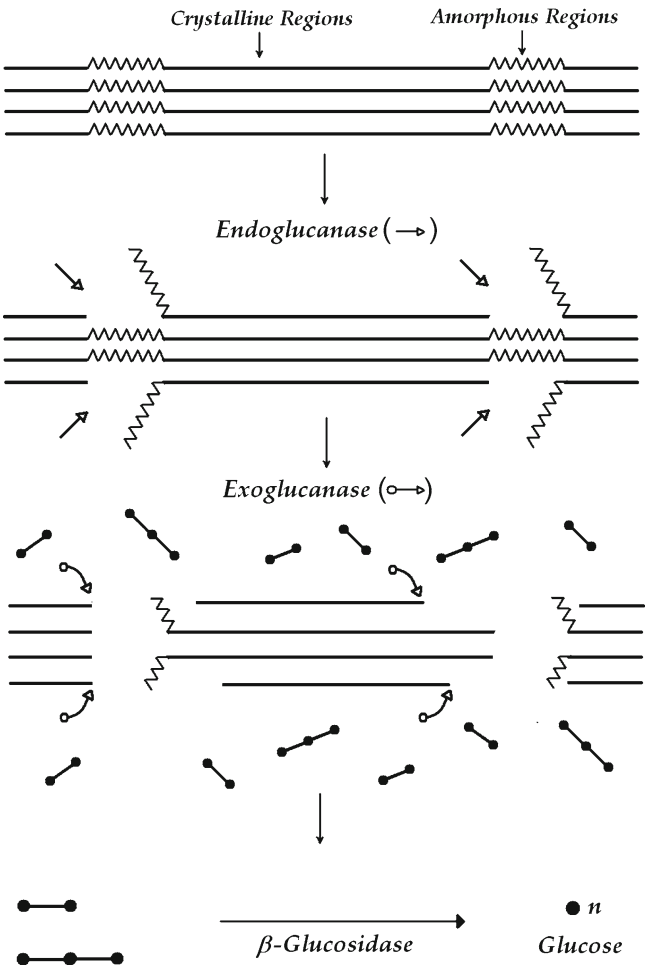

Fig. 1 Schematic representation of the sequential stages of the cellulolytic enzymes action

Hemicellulose is a heteropolysaccharide composed of various proportions of monosaccharide units of D-xylose, D-mannose, D-galactose, and L-arabinose (Polizeli et al. 2005). The hemicellulose molecules can be composed of branched chains with *O*-acetyl groups, D-glucuronic acid, 4-*O*-methyl-α-D-glucuronopyranosyl acid, D-galacturonic acid, ferulic acid, and *p*-coumaric acid. Classes of hemicellulose are usually named according to the main sugar unit. Hydrolyzed polymer which yields only xylose is named xylan; in the same sense, hemicelluloses include mannans, glucans, arabinans, and galactans.

In nature, wood hemicelluloses are amorphous complex structures made of more than one polymer and the most frequent are mannan (galactoglucomannans, glucomannans), glucuronoxylans, arabinogalactans, and arabinoglucuronoxylans. Then, the hydrolysis of β-1,4 linkages from glucomannan yields glucose and mannan. The hydrolysis of β-1,4 linkages from galactoglucomanan produces both manann and glucomanann and α-1,6 galactosyl residues linked. Because the amorphous morphology, hemicelluloses are partially soluble in water. The amount of each component varies from species to species and even among parts of the same plant. Sugarcane cell wall, for instance, is composed of arabinoxylans, but also of polysaccharides β-glucans, mannans, and pectins with neutral branching galactan (Silva 2005).

Xylans are considered the principal class of hemicelluloses and contribute to the total dry weight of angiosperms, as sugarcane, with 15–30%. Xylans are linear polymers of β-D-xylopyranosyl units (xylose) linked by β-(1-4) glycosidic bonds (Fig. 2). Depending on the origin of xylan the chain has substituent groups in the polysaccharide backbone. These include acetyl groups, 4-*O*-methyl-α-D-glucuronopyranosyl units and α-L-arabinofuranosyl groups. Angiosperm xylans have a high rate of

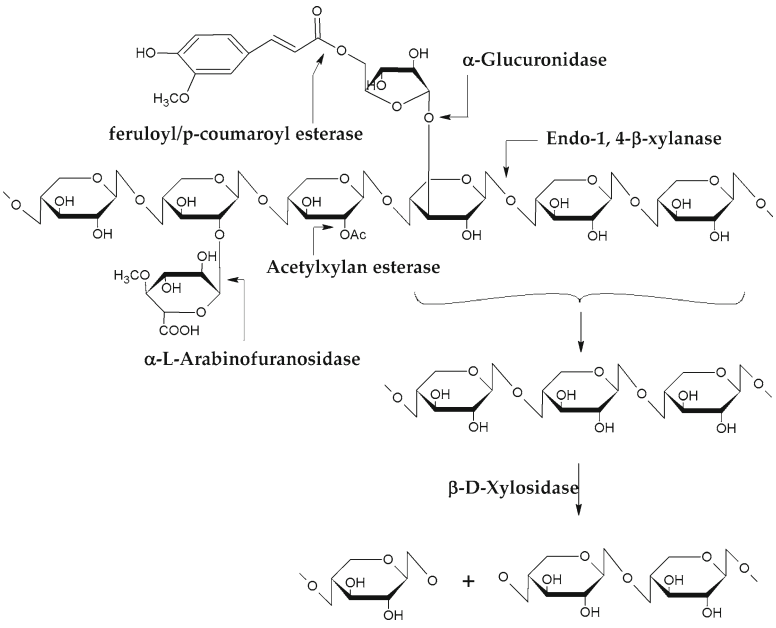

Fig. 2 Schematic representation of a xylan molecule and of the xylanolytic enzymes

substitution (70–80%) by acetyl groups, at position 2 and/or 3 of the β-D-xylopyranosyl, conferring to xylan its partial solubility in water. Subsequently, the lateral chains of xylan determine its solubility, physic conformation and the reactivity of the molecule of xylan with hemicellulosic components and, therefore, greatly influence the way and extension of its enzymatic hydrolysis (Polizeli 2009).

Lignin is a highly crossed-linked and stereochemically complex macromolecule of the cell wall which is formed from polymerization of phenylpropanoid precursors, as shown in Fig. 3. The synthesis of lignin starts with the deamination of L-phenylalanine by phenylalanine ammonia-lyase resulting into cinnamic acid. A series of enzymatic hydroxylations and methylations leads to the formation of *p*-coumaric acid, caffeic acid, ferulic acid, 5'-hydroxyferulic acid and sinapyl acid. Furthermore, reduction provides the monolignols: *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, which differs in the number of methoxyl groups on the aromatic ring. The monolignols are transported

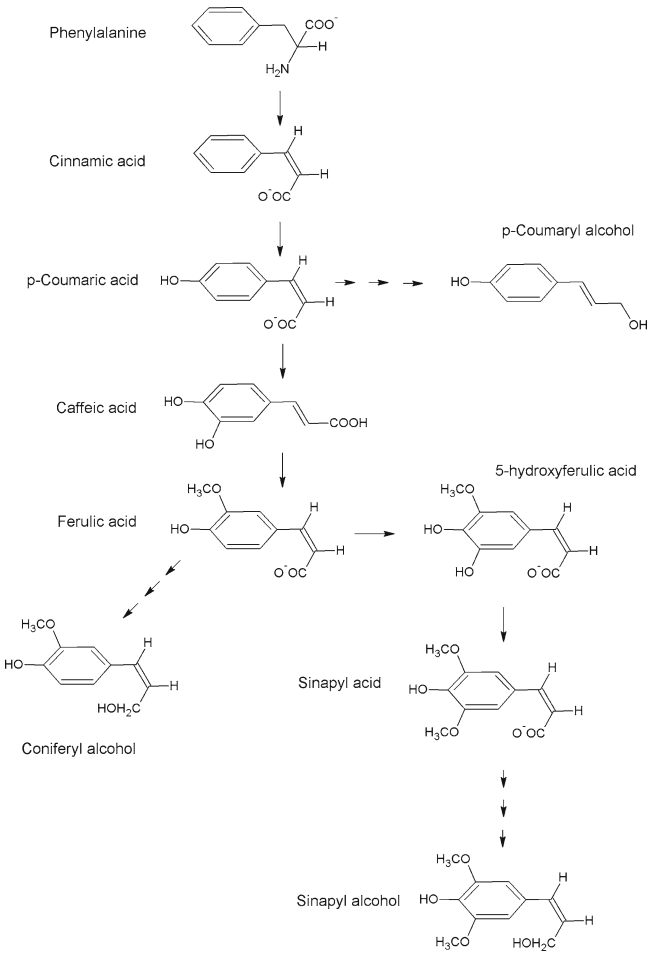

Fig. 3 Schematic representation of the formation of lignin precursors: *p*-coumaryl, coniferyl alcohol and sinapyl alcohol

from the cytosol, where they are synthetized as glucosides, to the cell wall, where the lignin polymerization takes place. The glucose is added to the monolignol to make them water soluble and to reduce their toxicity. After the transporting of glucose is its subsequently removal, the monolignols can couple with each other randomly, polymerizing the lignin to form a complex and irregular crossed-linked network structure.

Thus, lignin is considered one of the hardest organic polymers to degrade due to its insolubility, high molecular weight, and nonlinear and nonuniform structure. Different production plants use different monolignols. The total content of phenolic compounds in the prehydrolysated sugarcane bagasse corresponds to phenylpropanoid derivatives, such as *p*-coumaric and ferulic acids, accounting for more than 50% of the phenols identified by steam explosion) prehydrolysates (Martin et al. 2007).

6 Cell Wall Degrading Enzymes

The diversity and heterogeneity of the polysaccharides of cell wall structure contribute to a large extent to the difficulty found in enzymatic determinations. In this context, according to its chemical structure and the substituent groups of the lateral chain, the xylan and the overlapping sheath of the lignin are covalently bound. Ferulic acid tends to be associated with hemicellulose and *p*-coumaric acid seems to be generally esterified to lignin (Fig. 4). The covalently linked layers of xylans

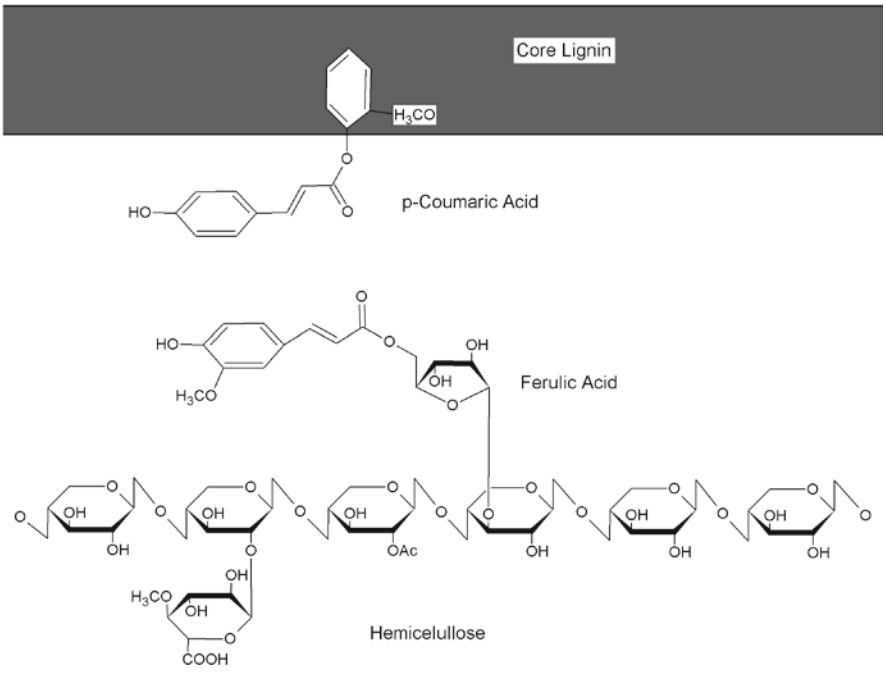

Fig. 4 Interaction of *p*-coumaric and ferulic acids to lignin of cell wall

and lignins and their noncovalent interaction with cellulose are important for the maintenance of the integrity of the cellulose in situ, and consequently, for the protection of the fibers against the action of cellulase. The xylan ramification points have an important function because they may cause steric hindrance. They prevent the formation of the enzyme-substrate complex; alter the xylan structure changing its solubility, and also change the configuration and the linking of phenolic components in the lignin (Polizeli 2009).

Therefore, complete cell wall hydrolysis demands enzymes that hydrolyze the branches (lateral chains), and synergistically contribute to the hydrolysis of the polysaccharide main chains.

7 Microbial Cellulose Degradation

Cellulose is degraded into glucose or low molecular weight soluble saccharides. This is due to the action of a multienzymatic system, which consists of three major components:

(1) Endoglucanase or endo 1,4- β-glucanase (EC 3.2.1.4) acting randomly on 1,4-β-linkages. It prefers to degrade amorphous (noncrystalline) rather than crystalline cellulose.
(2) Exoglucanase or exo-1,4-β-glucanase (EC 3.2.1.91, exo-1,4-β-D-glucan cellobiohydrolase; (CBH); CBH releases both glucose and cellobiose units from the nonreducing ends of cellulose chains.
(3) β-glycosidases or 1,4 – β-glycosidases (EC 3.2.1.21) hydrolyze main cellobiose to glucose, and cellobionic acids to glucose and gluconolactone.

The three enzymes act synergistically in the decomposition of bioemass and in a sequence of events. Endoglucanases randomly disrupt cellulose amorphous regions while exoglucanases act on exposed chain ends by splitting off cellobiose which is subsequently hydrolyzed by β-glucosidase into glucose (Fig. 1). β-glycosidases are the components which regulate the velocity of the process because endo- and exocelulases are inhibited by their product, glucose.

Cellulases are preferentially produced from wood decaying basidiomycetes as *Phanerochaete chrysosporium* (Igarashi et al. 2008), *Trametes versicolor* (Lahjouji et al. 2007), *T. hirsute* (Nozaki et al. 2007), *Ceriporiopsis subvermispora* (Magalhães et al. 2006), *Agaricus bisporus* (De Groot et al. 1998), *Volvariell volvacea* (Ding et al. 2006), and *Schizophyllum commune* (Henrissat et al. 1989). However, as reported by Henrissat et al (1989), the bacteria *Clostridium thermocellum*, *Erwinia chrysanthemi, Bacillus sp., Cellulomonas fimi, Streptomyces sp, Pseudomonas fluorescens*, and *Cryptococcus albidus* also produce cellulases in high levels. Filamentous fungi as *Hypocrea jecorina (Trichoderma reesei)* (Schmoll and Kubicek 2003) and in minor extension *Aspergillus sp* (Dedavid and Silva et al. 2009) and *Penicillium* (Camassola and Dillon 2007) are also reported as cellulase producers.

Phanerochaete chysosporium also produces oxidative enzymes that also play an important function in the cellulose degradation. The flavoprotein cellobiose:quinine oxidoreductase (EC 1.1.5.1) reduces the quinines and phenoxy radicals which were produced during lignin degradation, in the presence of cellobiose, which is oxidized to cellobio-α-lactone. The flavoheme protein, cellobiose oxidase (EC 1.1.3.25) is another enzyme which oxidizes cellobiose and higher cellodextrins into their corresponding onic acids using molecular oxygen. Both enzymes have been shown to bind at microcrystalline cellulose while retaining enzyme activity. Some authors (Wood and Wood 1992) have suggested that cellobiose:quinine oxidoreductase is not an independent enzyme, but a breakdown product of cellulose oxidase instead, which acts as a link within the lignin–cellulose degradation processes.

8 Microbial Hemicellulose Degradation

Due to the heterogeneous structure of hemicelluloses, their complete degradation requires a microbial enzymatic system with several enzymes, acting on the main chain or the lateral chain due to the presence of substituent groups. Important enzymes which act in the main chain are: endo-1,5-α-L-arabinase (arabinan endo-1,5-α-L-arabinosidase EC 3.2.1.99) acting in the endohydrolysis of 1,5-α-arabinofuranosidic linkages in 1,5-arabinans (Sakamoto et al. 2003); β-D-mannanase (mannan endo-1,4-β-mannosidase, EC 3.2.1.78), perform randoming hydrolysis of 1,4- β-D-mannosidic linkages in mannans, galactomannans and glucomannans (Luo et al. 2009); β-D-mannosidase (β-D-mannoside mannohydrolase EC 3.2.1.25) hydrolyzing terminal, nonreducing beta-D-mannose residues in beta-D-mannosides (Athanasopoulos et al. 2005; Tanaka et al. 2009), and enzymes of the xylanolytic system, as described above. Mannanase is reported in microorganism as fungi (Luo et al. 2009), bacteria (Tanaka et al. 2009), and heterologous expression in yeast (Setati et al. 2001). Fungal β-D-mannanase has different characteristics in relation to molecular mass, isoelectric point, pH and temperature.

The main xylanolytic enzymes are endo-1,4-β-xylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) and β-D-xylosidase (1,4-β-D-xylan xylohydrolase; EC 3.2.1.37) (Fig. 2). Endo-1,4-β-xylanase cleaves glycosidic bonds in the xylan backbone, producing xylooligosaccharides of variable lengths, resulting in a reduction of the Degree of Polymerization of the substrate. The xylan is not attacked randomly, since the bonds selected for hydrolysis depend on the chain length, the degree of branching and the presence of substituents (Polizeli et al. 2005; Polizeli 2009). Initially, the main products of xylanase action are xylose oligomers, but at a later stage, small molecules such as the mono-, di-, and trisaccharide of β-D-xylopyranosyl may be released. The accumulation of these short oligomers of xylose may inhibit the endoxylanase activity.

The β-D-xylosidase hydrolyses that these short oligomers formed by endo-1,4-β-xylanase action removing the cause of inhibition and increasing the efficiency of xylan hydrolysis (Polizeli et al. 2005; Polizeli 2009). The best substrate for β-xylosidase is xylobiose. Its affinity for xylooligosaccharides is inversely

proportional to their Degree of Polymerization. β-xylosidases are also able to cleave artificial substrates, such as *p*- and *O*-nitrophenyl-β-D-xylopyranoside. A good example of stable β-xylosidase is the enzyme from *Aspergillus phoenicis*, which retains 100% of its activity after 4 h at 60°C or 21 days at room temperature (Rizzatti et al. 2001). Transxylosylation activity has also been detected in fungi, resulting in higher molecular weight products than the original substrates (Kurakabe et al. 1997).

The association of xylanases results in a "xylanosome," a term applied to structures observed as protein aggregates with many subunits. It is an analog structure to cellulosome that is a multienzyme system that may mediate the adhesion of the bacterium to cellulose (Polizeli et al. 2005; Jiang et al. 2004, 2006; Deng et al. 2005). Xylanosomes have very high molecular weights (500–600 kDa) and consist of more than 10 proteins with xylanolytic activity, several of which are endoxylanases. In a few microorganisms, a cellulosome may be associated with a xylanosome, forming large complexes responsible for the hydrolysis of both cellulose and xylan (Sunna and Antranikian 1997).

Other microbial enzymes act on xylan branched groups (Fig. 2):

(1) Acetylxylan esterase (Li et al. 2008) (EC 3.1.1.72), catalyzes the hydrolysis of acetyl groups from polymeric xylan, acetylated xylose, acetylated glucose, α-naphthyl acetate, and *p*-nitrophenyl acetate.
(2) α-D-Galactosidase (Simerska et al. 2007) (α-D-galactoside galactohydrolase, EC 3.2.1.22), hydrolyses of terminal, nonreducing α-D-galactose residues into α-D-galactosides, including galactose oligosaccharides, galactomannans, and galactohydrolase.
(3) α-Glucuronidase (Tenkanen and Siika-aho 2000) (xylan-α-D-1,2-(4-*O*-methyl) glucuronohydrolase, EC 3.2.1.131), that hydrolyses α-D-1,2-(4-*O*-methyl) glucuronosyl links in the main chain of hardwood xylans.
(4) Feruloyl/*p*-coumaroyl esterase (Hermoso et al. 2004) (4-hidroxy-3-methoxycinnamoyl-sugar hydrolase, EC 3.1.1.73) that cleaves ester bonds on xylan between arabinose and ferulic acid and/or arabinose and *p*-coumaric acid side-groups.
(5) Two types of α-arabinofuranosidase, with distinct modes of action: the most common, exo-α-L-arabinofuranosidase (Rahman et al. 2003) (α-L-arabinofuranoside arabinofurano-hydrolase, EC 3.2.1.55), degrades *p*-nitrophenyl-α-L-arabinofuranosides and branches arabinans, whereas endo-1,5-α-L-arabinase (Takao et al. 2002) (arabinan endo-1,5-alpha-L-arabinosidase, EC 3.2.1.99) only hydrolyzes linear arabinans.

The xylanases were originally classified into two families of glycosyl hydrolases, denominated F and G, or 10 and 11 (Henrissat and Bairoch 1993; Sapag et al. 2002), respectively. This classification is based in the structural homology of the hydrophobic regions, deduced from 2D representations of the primary sequences. More recently Collins et al. (2005) reported the existence of enzymes with xylanase activity also belonging to families 5, 8, and 43.

The family F/10 consists of a cellulose-binding domain and a catalytic domain, which is a TIM barrel domain, interconnected by a flexible spacer and a substrate-binding domain. The family G/11 is composed of highly specific endoxylanases of low Mw,

with 40–90% sequence identity, with different pIs, thermostabilities, pH profiles, structures, and catalytic properties. According to Törrönen and Rouvinen (1997), besides these homology-based classes, xylanases could be further divided into those with alkaline and those with acidic pI, but some microorganisms produce both types, e.g., *Aspergillus* and *Trichoderma* sp. This resulted in a further classification of the xylanases in classes such as A, B, C, D, 1, 2, 3, I, II, III (Kulkarni et al. 1999a, b).

Aiming biotechnological applications, the ideal source of xylanase would be one that produces an adequate amount of each of the enzymes composing the xylanolytic system. The synergistic interactions among the xylan-degrading enzymes enhance the susceptibility of the xylan to the attack of endoxylanases. Among microbial sources, filamentous fungi are especially interesting as they secrete the enzymes into the medium, and their xylanase levels are higher than those in yeasts and bacteria.

Cloning, sequencing, and expression of xylanase genes in *Escherichia coli* has been performed for microorganisms of different genera (Kulkarni et al. 1999b; Liu et al. 1999). The xylanases in bacteria are restricted to the intracellular or periplasmic fractions (Schlacher et al. 1996), although extracellular activity has also been reported (Karlsson et al. 1998; Ebanks et al. 2000). Furthermore, enzymes expressed in bacteria are not subjected to posttranslation modifications such as glycosylation.

Besides bacteria, xylanases have also been expressed in the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris. Saccharomyces cerevisiae* is an attractive host for the expression of heterologous xylanases (Romanos et al. 1992). It provides an efficient posttranslational processing such as glycosylation, proper folding of the proteins, proteolysis (Sa-Pereira et al. 2003). Besides being a host that secretes only some proteins, *S. cerevisiae* expressed proteins are easier purified (Das and Shultz 1987). On the other hand, heterologous gene expression by the methylotrophic yeast *Pichia pastoris* is a useful alternative when scaling up to industrial process and has several advantages over *Saccharomyces cerevisiae* because of high secretion efficiency, high cell densities attained in inexpensive culture media, and the relative ease of scaling up the industrial process (Cregg 1999). It is a particularly attractive expression host due to the availability of strong and regulatory promoters that are involved in methanol metabolism (Tsai and Huanga 2008).

Filamentous fungi are attractive hosts for protein expression because of their natural ability to secrete large amounts of proteins into the medium. Fungi dominating the market as expression hosts are *Aspergillus niger* (Levasseur et al. 2005), *Aspergillus nidulans* (Perez-Gonzalez et al. 1998), *Aspergillus oryzae* (Kimura et al. 2002), and *Trichoderma reesei* (Mantyla et al. 2007). Most of the xylanase genes have been expressed in fungi under homologous systems.

Xylanases may be induced either by incubating microorganisms in submerged liquid culture (SbmF) (Rizzatti et al. 2004) or on different solid substrates (SSF) as wheat bran (Betini et al. 2009), rice bran (Dos Santos et al. 2003), sugarcane bagasse (Dos Santos et al. 2003; Sandrim et al. 2005), and others. The induction of the xylanolytic system is supposed to occur through physical contact between some recognition site on the cell surface and the inducer. Most fungal strains are induced by xylan, and carbon catabolite repression by glucose is reported as well. Relatively low levels of constitutive xylanases may be responsible for the initial hydrolysis of

xylan, producing xylotriose, xylobiose, and/or xylose (Polizeli et al. 2005; Polizeli 2009). These oligosaccharides are transported into the cell with the aid of β-xyloside permeases, and then trigger the transcription of the xylanolytic system genes. Permease activity is reduced by the presence of glucose, but it is very efficient in the presence of the xylanolytic inducers.

In filamentous fungi, carbon catabolite repression is mediated by the protein CREA (transcription repressor). Few studies detail the transcriptional regulation of xylanase and the participation of the cAMP in the repression by glucose. Morosoli et al. (Morosoli et al. 1987) report the xylanase from *Cryptococcus albidus* is induced either by xylan or β-methylxyloside, a nonmetabolizable inducer, and that xylose represses the production of the enzyme. In this microorganism, addition of exogenous cAMP elicits a 1.5–2-fold increase in xylanase production in the presence of the inducer, but does not relieve the repression caused by xylose. These authors suggest that a 15-nucleotide sequence located upstream from the xylanase gene could be part of a cAMP regulatory site. Rizzatti et al. (2008) reported that the xylanase of *Aspergillus phoenicis* was induced by xylan, xylose, and β-methylxyloside but repressed by the addition of glucose. The glucose repression was alleviated by addition of cAMP or dibutyryl-cAMP. These physiological observations were supported by a Northern analysis using part of the xylanase gene *ApXLN* as a probe. Gene transcription was shown to be induced by the same inducers, and was repressed by glucose, where the repression was partially relieved by the addition of cAMP or dibutyryl cAMP.

Catabolic repression in *Aspergillus nidulans* and *A. niger* is also described by Ruijter and Visser (Ruijter and Visser 1997). These authors suggest that the CREA protein plays a major role in carbon repression by binding to specific sequences in the promoter of many target genes, and inhibiting their transcription. More recently, it was isolated a transcriptional activator of xylanases (XlnR) from *Aspergillus niger* (van Peij et al. 1998) and a transcriptional activator (AoXlnR) in *Aspergillus oryzae* which is XlnR homolog. Northern blot analysis reveals the controls of the expression of genes encoding xylanolytic enzymes (Marui et al. 2002). Repression of xylanolytic genes by glucose has been assigned to the protein CREA in *A. niger* (de Graaff et al. 1994)) and to its homolog Cre1 in *Trichoderma reesei* (Strauss et al. 1995). CREA protein modulates the expression of XlnR induced by xylose in *A. niger* (de Vries et al. 1999) and *A. nidulans* (Prathumpai et al. 2004).

In general, fungal endoxylanases have optimum activity and stability in the range of 40–75°C and pH 4.0–6.5 although extremophile bacteria produce endoxylanases with an optimal temperature higher than 80°C (Polizeli 2009). Endoxylanases are usually small glycoproteins (6–50 kDa), with multiplicity of forms, being that in some cases three or more enzymatic isoforms from a single culture (Rizzatti et al. 2004). A number of factors may be responsible for this phenomenon, such as differential processing of mRNA, posttranslation modifications (glycosylation and self-aggregation) or proteolysis. Multiple endoxylanases can also be expressed by distinct alleles of one gene, or even by completely separate genes (Polizeli et al. 2005).

9 Microbial Lignin Enzymes

Lignin is degraded by different combinations of extracellular oxidative enzymes found mainly in ligninolytic cultures of white-rot fungi. Here, we comment briefly about Lignin Peroxidase (LiP); Manganese Peroxidase (MnP), and laccase.

Lignin Peroxidase: (1,2-bis [3,4-dimethoxyphenyl] propane-1,3-diol:hydrogen-peroxide oxidoreductase, EC 1.11.1.14) belongs to the family of oxidoreductases specifically those acting on a peroxide as acceptor (peroxidases). It is an extracellular hemeprotein, with an unusual high redox potential and low optimum pH. It reacts with a wide variety of lignin related compounds end even unrelated molecules. The chemical reaction catalyzed is:

$$\begin{aligned}&1,2-\text{bis}(3,4-\text{dimethoxyphenyl})\text{propane}-1,3-\text{diol}+H_2O_2\\&\rightleftharpoons 3,4-\text{dimethoxybenzaldehyde}+1-(3,4-\text{dimethoxyphenyl})\\&\text{ethane}-1,2-\text{diol}+H_2O.\end{aligned}$$

Manganese Peroxidase: (Mn[II]: hydrogen-peroxide oxidoreductase, EC 1.11.1.13) is another type of heme peroxidase. This oxidoreductase shows a strong preference for Mn(II) and acts on a peroxide as acceptor (peroxidase). The product Mn(III) forms a complex with organic acids and diffuses away from the enzyme to oxidize other materials, such as lignin. The chemical reaction is:

$$2\text{Mn(II)}+2H^{+}+H_2O_2 \rightleftharpoons 2\text{Mn(III)}+2H_2O$$

The redox potential of the Mn peroxidase is lower than that of lignin peroxidase and it has only shown capacity to oxidize in vitro phenolic substrates (Rahi et al. 2009), which are oxidized into phenoloxy radicals, further reacting through demethylation, alkyl-phenyl cleavage, Cα-oxidation, or Cα-Cβ cleavage. Manganese peroxidase seems to be more widespread among white-rot fungi than lignin peroxidase.

Laccase (EC 1.10.3.2): is a copper-containing oxidase found in many plants and microorganisms and it does not require peroxidase, H_2O_2. These enzymes oxidize phenol and similar compounds, forming phenoxy radicals. However, in presence of the artificial substrate ABTS (2,2-azinobis(3-ethylbenzothiazoline-5-sulphonate) or some other synthetic mediators, laccase can also oxidize certain nonphenolic compounds, veratryl alcohol and Mn(II). Laccase has the capability to both depolymerize and polymerize lignin model compounds. It is proposed that laccases play a role in the formation of lignin by promoting the oxidative coupling of lignols.

10 Microbial Enzymes and Thermophile

Within an evolutionary context, thermophiles (Cooney and Emerson 1964) probably emerged from a mesophile branch as a consequence of many mutations which allowed their growth in hot environments and limited it in cold temperatures. Glycosylation

itself could not explain the ability for these organisms to survive in such different conditions. Punctual site mutations in the amino acid sequences can also change this characteristic. As for hyperthermophiles, the thermal stability of enzymes and other proteins is also due to saline bridges. Furthermore, there are hydrophobic bounds in the inner side of the proteins which favor the resistance to the unfolding and denaturation of the molecule in aqueous medium (Madigan et al 2000).

Thermophiles and hyperthermophile cells also present changes which help keep their thermal characteristic. These changes are mainly in the biochemical machinery and more heat-stable cell structures. The membranes of thermophiles are rich in saturated fatty acids which turn the medium into a much more hydrophobic environment helping the maintenance of membrane stability. Hyperthermophiles, encompassing many organisms from the domain Archaea, do not present lipids with fatty acids in their membranes but isopropane, a compound constituted by repeated units of five carbons linked to glycerol phosphate.

Another curious fact is that hyperthermophiles increase its concentration of chaperon proteins drastically in each cell when submitted to high temperatures close to the maximum tolerant. Chaperone proteins aid other proteins protecting them and increase cell thermal resistance (Madigan et al 2000). Pyrodictium, an archaeum organism, can survive for 1 h at 121°C achieving a chaperone protein concentration of 80% out of total citoplasmatic proteins. Thermophiles exhibit an increase of heat shock proteins and trehalose in response to temperature increasing. This disaccharide interacts with the solvatation layer of the protein keeping the aggregation and denaturation of the molecule from happening.

Thermophiles are of great interest because they produce considerable quantities of enzymes (Bhat and Maheshari 1987; Latif et al. 1995) and they are more thermal stable when compared with similar ones produced by mesophiles. The high growing temperature also avoids contamination by undesirable microorganisms (Bhat and Maheshari 1987; Latif et al. 1995). *Scytalidium thermophilum* produces a mycelial β-glycosidase, which is activated by glucose or xylose at a concentration varying from 50 to 200 mM (Zanoelo et al 2004a). This kind of enzyme is greatly desirable in scarification processes because a high rate of hydrolysis could be attained without glucose inhibition. This same species of fungus is also found to produce a β-xylosidase tolerant to its product, xylose, in range until 200 mM (Zanoelo et al 2004b) and when grown in Reese minimum medium (Reese and Mandels 1963) added with rice straw as the only carbon source can produce high amounts of xylanases and only traces of cellulases (Gaur et al 2005), which is important to pulp production used in paper manufacturing.

11 Perspectives

The population growth and the increasing demand for fuels have intensified the research and development of utilization of renewable raw material in substitution of fossil sources. The advances in this area signalize that the exploitation of renewable

raw material, including residues, will revert this dependence on crude oil. Until now, corn and sugarcane broth were the major resources in the emerging cellulose-to-ethanol strategy for biofuels with technologies commercially established. The next step is the exploitation of the bagasse and straw of sugarcane for ethanol production.

Cellulose and hemicellulose together, represent approximately 70% of lignin–cellulosic materials. The potential bioconversion of these carbohydrates into biofuels is limited by association with aromatic constituents (lignin). Structural studies over the years have identified the limitation of fiber degradation imposed by aromatic barriers, e.g., coniferyl lignin appears to be the most effective limitation for biodegradation, existing in xylem cells of vascular tissues. Cell walls with syringyl lignin, e.g., leaf sclerenchyma, are often less intractable. On the other hand, ferulic and *p*-coumaric acids esterified into xylan constitute a major limitation for biodegradation of nonlignified cell walls. Nonchemical methods to improve bioconversion of the lignocelluloses include pretreatment of sugarcane bagasse with phenolic acid esterases, plant breeding to modify cell wall aromatics and separation and collection of aromatics to increase availability of carbohydrates for fermentation.

In addition, a better judgment of the regulatory system of the lignocellulosic enzymes is still limited. Great hopes are concentrated on technological advance, but there is also a search for new microorganisms, within the immense biodiversity of this planet, which may possess better adaptive characteristics in relation to temperature, pH of the medium and adaptability to low-cost substrates, hardly exploited up to now. These microorganisms responsible for lignincellulosic biodegradation also present ecological importance because they generate metabolic energy that maintains the flow of carbon, and their importance in the natural recycling of biomass is enormous. The huge potential of thermophiles, thermotolerant, and extremophile microorganisms should also be emphasized, considering their impact in several areas of application. On the other hand, we cannot forget the undeniable contribution of the advent of recombinant DNA technology and phylogenetic studies which can provide a better focus on wanted targets and minimize efforts by seeking closely related organisms sharing desirable characteristics. All of these alternatives should be considered to improve the value added to coproducts of bioconversion from sugarcane into biofuel.

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Chapter 9
Cellulase Engineering for Biomass Saccharification

Richard J. Ward

1 Introduction

The production of biofuel ethanol from sugarcane biomass could be significantly improved by depolymerization of cell wall cellulose to glucose for subsequent fermentation. Enzymatic hydrolysis of cellulose using a mixture of glucanases has been proposed as a possible technology to achieve this goal. It is likely that different biomass feedstocks will require specific enzymes, and this chapter examines the range of strategies which may be used for engineering cellulases to modulate their specificities and catalytic activities.

2 Cellulose and Cellulases

The production of biofuel ethanol from sugarcane saccharose is currently restricted to the exploitation of approximately 35% of the total energetic value of the raw material. The yield could be significantly improved by a technology that mobilizes the remaining 65% of the energetic value that is present in the form of lignocellulose, the principal component of the plant cell wall, and which comprises three principal polymers; cellulose, hemicellulose, and lignin. Cellulose is a polysaccharide formed by linear chains of D-glucose linked by β-1,4-bonds, to form the disaccharide cellobiose (4-*O*-(β-D-glycopyranosil-D-glucopyranose)) repeat unit. Bundles of cellulose chains are aligned to form organized fibrils in which the individual cellulose polymers participate in alternating crystalline and amorphous structures. The cellulose fibrils are stabilized by an extensive network of inter- and intramolecular hydrogen bonds that impart a combination of high tensile strength and low solubility in aqueous environments.

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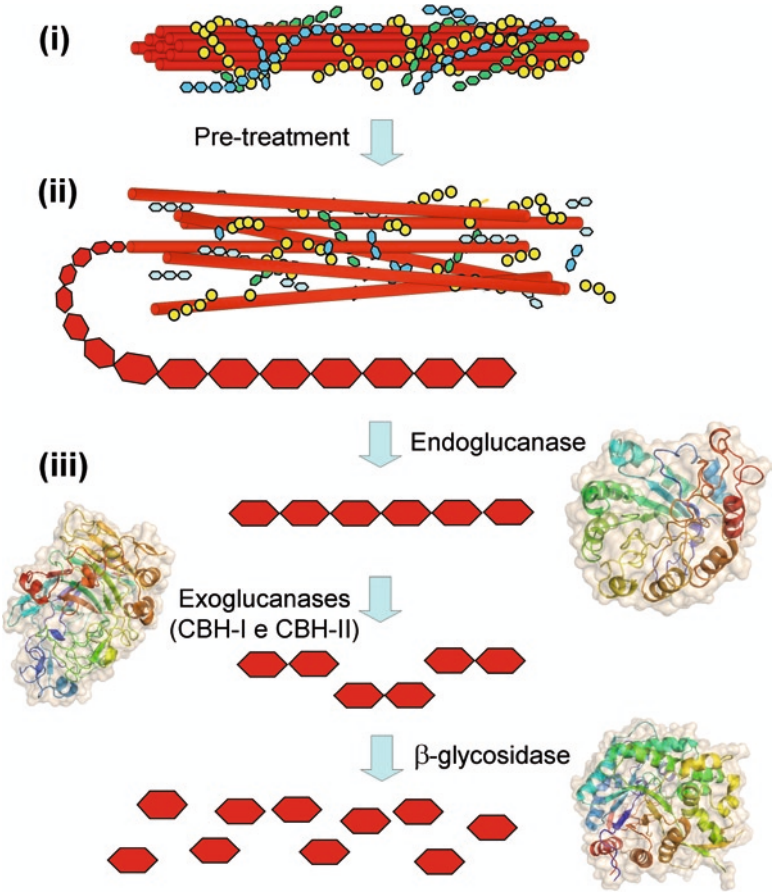

Fig. 1 Enzymatic hydrolysis of lignocellulose. (i) Cellulose fibers (in *red*) are embedded within a compound matrix comprised of hemicellulose (*light grey*), and pectin (*dark grey*), polymers. Prior to treatment by enzymes, the lignocellulosic material is typically subjected to pretreatment, for example, exposure to acids or vapor explosion. The breakage of covalent bonds in the hemicellulose and pectin polymers may be accelerated by xylanase and pectinase respectively. (ii) The pretreatment disorganizes the packing of the cellulose fibers, leaving them more susceptible to enzymatic attack. (iii) The cellulose fibers comprise bundles of cellulose polymers (glucose monomers are represented as *grey* hexagons); endo-1,4-β-glucanases (EC 3.2.1.4) cleave the cellulose polymers exposing reducing and nonreducing ends in the hydrolysis products. The reducing and nonreducing ends are further hydrolyzed by exoglucanases (or cellobiohydrolases CBH-I e CBH-II, EC 3.2.1.91) to release cellobiose, which is finally hydrolyzed to glucose monomers by β-glucosidase (EC 3.2.1.21). The structures shown are the catalytic domain of endocellulase Cel5 from *Thermobifida fusca* (PDB code 2CKS) (Berglund et al. 2007), the exocellulase Cel7A cellobiohydrolase (CBHI) from *Hypocrea jecorina* (PDB code 5CEL) (Divne et al. 1998) and the Cel1 β-glucosidase A (BglA) from *Thermotoga maritima* (PDB code 1OD0) (Zechel et al. 2003)

The individual cellulose polymers in lignocellulose are refractory to hydrolysis, which presents a major technological hurdle for the utilization of plant biomass as a source of fermentable sugars. Enzymatic hydrolysis coupled with a suitable pretreatment of the lignocellulose is a promising alternative and requires the synergistic action of a "cocktail" of hydrolases (see Fig. 1), culminating in the hydrolysis of cellulose polymers by three cellulolytic enzymes. These glycosidases hydrolyze the β-1,4 glycosidic bond between glucose monomers and include endo-1,4-β-glucanases (EC 3.2.1.4) which randomly hydrolyze glycosidic bonds in cellulose, exoglucanases (EC 3.2.1.91) which remove cellobiose (two glucose monomers joined by a glycosidic bond) from the reducing or nonreducing extremities of the cellulose polymer, and β-glycosidases (EC 3.2.1.21) which hydrolyze cellobiose to produce glucose.

A critical factor for the successful application of enzymes for biomass saccharification is the choice of suitable enzymes that present a high hydrolytic efficiency against cellulose. The identification and characterization of cellulases produced by microorganisms isolated from the environment, together with programs for improving enzyme production, has provided most of the cellulase preparations currently available. However, it is likely that viable cocktails will require formulations of enzymes with activities that are tailored for specific biomass feedstocks, and protein engineering strategies can alter the catalytic properties of enzymes to improve existing functions or even introduce novel properties. The focus of this chapter will be on the contribution by protein engineering to the current understanding of the molecular bases of key aspects of endo- and exocellulase activity, together with efforts to improve catalytic function for application in biomass saccharification.

3 Protein Engineering Strategies

It is paradigmatic that the three-dimensional structure of a protein determines its biochemical function, and knowledge of the spatial arrangement of the amino acids within the protein is the key to understand the molecular basis for correct molecular recognition, specific catalysis, and molecular flexibility that is essential for protein function. The analysis of three-dimensional structural models of proteins can predict their biological function, catalytic mechanism, the binding sites of substrates, inhibitors, and activators, and the regions on protein surfaces that interact with other biomolecules. The understanding of the molecular basis of activity is of key importance for the design of enzymes with modified functions.

3.1 Cellulase Engineering by Rational Design

The use of "rational" methods to plan structural alterations in proteins is widely used in protein engineering. A common strategy is site-directed mutagenesis of conserved residues identified by amino acid sequence comparison between

homologues from differing species, and this approach has been applied to reduce the pH optimum of the glucohydrolase family 5 (GH5 or Cel5 using the nomenclature introduced by Henrissat and Bairoch 1996) endoglucanase CelC from *Clostridium thermocellum* (Navas and Beguin 1992). Analysis of crystal structures can provide more subtle clues for mutational studies. For instance, the cellobiohydrolase II Cel6A from *Hypocrea jecorina* contains five carboxyl-carboxylate ion pairs, including the active site residues D175–D221. Mutagenesis of the remaining nonactive site residues to amide-carboxylate pairs (by E to Q or D to N substitutions) cumulatively increased the pH stability and increased the pH range of the catalytic activity (Wohlfahrt et al. 2003).

Analyses of the three-dimensional structure of the protein of interest may be coupled with molecular dynamics simulation, which yields important information with respect to the structural mobility of the protein including key residues involved in the maintenance of secondary and tertiary structures. An elegant example of rational design was based on a molecular dynamics simulation of the mesophilic Cel5 endo-1,4-β-glucanase from alkalophilic *Bacillus* sp. strain KSM-64 (Ozawa et al. 2001). It was proposed that thermostability would be improved by the N179K and D194K mutants that would result in the formation of intrahelical ion pairs with residues E175 and E190, respectively, and this prediction was confirmed experimentally by site-directed mutagenesis (Ozawa et al. 2001). The rational design strategy is most powerful when combined with cocrystallization studies with substrates and inhibitors to study catalytic mechanism, and the use of these techniques for engineering the catalytic machinery of cellulases is described in Sect. 3.

3.2 Random Mutagenesis – Directed Evolution

Recent advances in recombinant DNA technology in conjunction with techniques for automated high-throughput clone selection and screening have prompted the emergence of "directed evolution" (DE) methods as powerful strategies for engineering novel properties in proteins (Bloom et al. 2005; Rohlin et al. 2001; Ruller et al. 2008). In vitro DE techniques mimic the process of random mutation and natural selection that drives Darwinian evolution *in natura*. Various DE strategies have been successfully applied to alter a wide range of protein functions including thermal stability and catalytic efficiency (Arnold and Moore 1997; Arnold and Volkov 1999). Frequently, a population of protein variants is produced using error-prone PCR (epPCR), which introduces random mutations along the length of a given DNA sequence. Subsequently, those variants that display properties that are of interest are selected and identified by nucleotide sequencing. These selected sequences may then be submitted to repeated cycles of mutagenesis and selection in order to accumulate mutations that determine a given function of the protein. This strategy has been used to modulate the catalytic activity of cellulases, for example, epPCR of the family Cel12A endoglucanase III (EGIII) from *H. jecorina*

(formerly *Trichoderma reesei*) generated the N321T mutant that showed an optimal activity of pH 5.4 as compared to pH 6.0 for the wild-type enzyme. Subsequent site-directed mutagenesis of the same position showed the N321D mutant reduced pH optimum to 4.0, whereas the N321H mutant showed broader pH tolerance with no reduction in catalytic activity (Wang et al. 2005). Similarly, high-throughput screening of a mutant library of the family Cel6 endoglucanase cellulase F from the anaerobic fungus *Orpinomyces* PC-2 allowed selection of mutants with a reduced pH optimum (Hughes et al. 2006).

3.3 In Vitro Recombination – DNA Shuffling

An alternative strategy in DE is the use of in vitro recombination, or "DNA shuffling." In this case, selected DNA sequences are randomly fragmented using DNaseI, and recombined by annealing and extension of the DNA strands using self-primed PCR, followed by selection of clones with desired properties from a library of recombined fragments (Patten et al. 1997; Stemmer 1994). DNA shuffling is commonly used to recombine mutants selected from random libraries generated by epPCR, and using a combination of epPCR and DNA shuffling, the pH optimum of the Cel5A endo-β-1,4-glucanase II from *T. reesei* was increased from 5.8 to 6.2 (Qin et al. 2008).

In vitro recombination is a powerful tool in protein engineering, and the technique may be used to generate recombined DNA libraries from mixtures of genes that encode enzymes with desired catalytic properties. For instance, DNA shuffling of mesophilic and thermostable Cel5 alkaline endo-1,4-β-glucanases from *Bacillus* sp. strains KSM-64 and KSM-S237 identified K137, K179, and K194 as likely determinants of thermostability, predictions which were confirmed by introduction of these residues using site-directed mutagenesis of the mesophilic enzyme (Hakamada et al. 2001). In another example, the thermostability of family Cel5 endoglucanase EngB from the cellulosome *Clostridium cellulovorans* was increased sevenfold by DNA shuffling of the genes for EngB and the highly similar noncellulosomal EngD from the same organism (Murashima et al. 2002).

With improved understanding of protein architecture and protein structure/function relationships, recent applications of in vitro recombination for engineering cellulases have become more sophisticated. A series of chimeric fungal class II cellobiohydrolases (CBH II cellulases) has been created by recombining DNA fragments that encode conserved internal structural motifs from three homologous fungal CBH II cellulases (Heinzelman et al. 2009). The three parent enzymes had pair-wise amino acid sequence identities ranging from 64 to 82%, and after recombination, the 15 most stable chimeric enzymes showed between 7 and 15°C increases in activity temperature optimum (Heinzelman et al. 2009). This example demonstrates that the recombination of structurally conserved motifs is a powerful strategy for incorporation of sequence diversity and suggests how natural biodiversity may be harnessed for engineering biocatalysts with desired properties.

3.4 Screening Randomized DNA Libraries

In all DE strategies, large numbers of clones must be evaluated in order to identify phenotypes showing the property of interest, and the full potential of DE strategies can only be realized when coupled to high-throughput screening techniques. Halo formation assays carried out on Petri dishes containing solid culture medium impregnated with cellulose require evaluation of single colonies and can be used to screen 10^4 clones, and this number may be increased to 10^6 by using automated techniques (Percival Zhang et al. 2006). Alternatively, cell surface display of enzymes is a powerful method for screening DNA libraries created by either epPCR or DNA shuffling. Bacterial surface display has been used to screen a library of cellulase genes generated by DNA shuffling and fused to the ice nucleation protein gene to select variants with improved growth on carboxymethylcellulose (CMC) substrates. The majority of amino acid substitutions from bacterial colonies demonstrating rapid growth on CMC plates were located in the catalytic domain (CD) of the enzyme, and rate enhancements of up to fivefold against the substrate could be achieved (Kim et al. 2000).

More recently, a short oligosaccharide has been used as a linker molecule to join the subunits of a transcription factor heterodimer designed to regulate lethal gene expression in the yeast *Saccharomyces cerevisae*. Hydrolysis of the oligosaccharide by heterologous cellulases expressed in these cells separated the transcription factor subunits, resulting in loss of lethal gene transcription and cell survival. Approximately 10^8 clones from a recombinant library of *Erwinia carotovora* CelN, CelA, and CelV cellulases were evaluated, and two variants were selected showing 3.7- and 5.7-fold increases in catalytic efficiency in comparison with the control cellulase CelN (Peralta-Yahya et al. 2008). By altering the sugar sequence in the linker molecule, this technology could easily be adapted to screen for a wide range of substrate-specific cellulases.

4 Engineering of Cellulase Properties

4.1 Cellulases Contain Substrate Binding and Catalytic Domains

As early as 1950, “a splitting of the cross linkages … in native cellulose” by cellulases was suggested as initial stage in hydrolysis and was recognized as being distinct from the catalytic cleavage of the glycosidic bonds of the polymer (Reese et al. 1950). Although the weaker hydrogen-bonded nature of these cellulose “cross-linkages” was not appreciated at the time, the mechanism through which individual cellulose polymers are separated from the cellulose fiber bundles and presented to the catalytic site of cellulolytic enzymes remains a key question.

Experiments using limited proteolysis by papain identified two functional domains in the family Cel7A cellobiohydrolase I (CBH I) from *H. jecorina*. Loss of

a 10 kDa glycosylated carboxy-terminal domain eliminated both binding and activity against insoluble crystalline Avicel, whereas the remaining 56 kDa core protein maintains activity against soluble substrates (Van Tilbeurgh et al. 1986). Subsequent studies yielded broadly similar results in other cellulases (Gilkes et al. 1988; Tomme et al. 1988), and although the use of proteolytic enzymes resulted in some nonspecific cleavage and uncertainty as to the boundaries between the cellulose binding and CDs, the concept of the modular nature of these enzymes was firmly established.

The appreciation of the modular nature of cellulases coincided with the development of recombinant DNA techniques, and as molecular cloning methods became more widespread, the domain structure of a range of cellulases could be studied unambiguously. Expression of a truncated form of the family Cel45 endoglucanase (EGB) from *Pseudomonas fluorescens* comprising only the C-terminal region did not bind Avicel yet retained catalytic activity, demonstrating that the N-terminal region is a cellulose-binding domain and is distinct from the CD (Gilbert et al. 1990). In addition, independent functions were demonstrated for the N-terminal CD and C-terminal crystalline cellulose-binding domain of the *Erwinia chrysanthemi* Cel5A endoglucanase (EGZ) (Py et al. 1991). Furthermore, residues 330–499 in the C-terminus of the family Cel5 endoglucanase from *Bacillus subtilis* PAP115 were shown to determine binding to amorphous cellulase, but did not influence activity against soluble CMC–cellulose (Hefford et al. 1992).

These studies confirmed the modular nature of the cellulases and also demonstrated that the non-CD that determined binding to crystalline cellulose substrates could be located at either the N- or C-terminal region of the enzyme. Furthermore, fully functional cellulose-binding and catalytic polypeptides could be expressed separately, showing the functional independence of the two domains. With these facts firmly established, the stage was set for the detailed study of the individual domains and created the possibility to modulate the specific functions of each domain of the enzymes.

4.2 The Cellulose-Binding Domain – An Example of a Carbohydrate-Binding Module

The carbohydrate-binding modules (CBM) are non-CDs that have widespread distribution and function in the binding of a wide variety of proteins to their cognate carbohydrates. The CBMs determine the targets for glucanase activity against specific substrates in various states, including insoluble polymers in the form of aggregates, on exposed polysaccharides or short-chain length oligosaccharides. Given this diversity, a unified nomenclature based on observed functions has proven problematic, and on the basis of the topology of the polysaccharide-binding surface, three types of CBM have been proposed (Boraston et al. 2004).

The “surface-binding” Type A CBM presents a flat exposed binding surface, which is compatible with binding to the essentially planar surfaces presented by

crystalline polysaccharides. In contrast, the "glycan-chain-binding" Type B CBMs show a recessed binding cleft that binds specific polysaccharides, and in this case, the binding specificity for the CBM and the CD shows strong convergence. Finally, the "small sugar-binding" Type C CBMs demonstrate the lectin-like binding to mono-, di-, or tri-saccharides and lack the extended binding site found in CBM types A and B (Boraston et al. 2004). Within these three CBM types are seven structural fold families which encompass the 54 CBM families known to date. By far the largest number are the CBMs with a β-sandwich fold with representatives distributed in types A, B, and C, whereas three other structural folds are exclusive to Type A and the remaining threefolds are found only in type C (Boraston et al. 2004). As new CBM candidates are discovered, this number of families is likely to increase, and CBM classification updates can be found on the Carbohydrate-Binding Module Family Server (http://www.cazy.org/fam/acc_CBM.html) (Cantarel et al. 2009).

4.3 Binding Models – What Type of Intermolecular Interaction is Important?

The essential role played by the CBMs in binding cellulases to crystalline cellulose substrates is well established, yet raises the question as to whether the plethora of CDM motifs reflect underlying variability in the mode or cellulose binding, or whether common principles underlie the structural diversity. Early mutagenesis studies of the CBM of the family Cel6 endo-β-1,4-glucanase (CenA) from *Cellulomonas fimi* showed that mutants W14A and W68A reduce the affinity of the enzyme for cellulose substrate (Din et al. 1994). Furthermore, mutation of the conserved tyrosine Y492 of the CBM of the family Cel7 cellobiohydrolase (CBHI) from *H. jecorina* reduced cellulose-binding affinity (Reinikainen et al. 1995). This study also showed that high ionic strength increased catalytic activity, suggesting that hydrophobic forces are important for CBM binding to the cellulose.

Since the publication of these early reports, the importance of aromatic residues in the binding of various CBMs to crystalline cellulose has been repeatedly demonstrated (Kataeva et al. 2001; Simpson and Barras 1999; Urbanowicz et al. 2007), and the crystal structures of CBMs in complexes with oligosaccharides support the idea that stacking of aromatic residues to bound sugar rings is a conserved mode of interaction (Flint et al. 2004; Machovic and Janecek 2006). In comparison, mutagenesis of polar residues located in the same protein surfaces that form the protein/polysaccharide interface have no effect on the binding of the CBM to the cellulose. For example, whereas residues W18, W43, and W44 are important in cellulose binding of the CBM of the Cel5 endoglucanase Z from *E. chrysanthemi*, mutations of Q22 and E27 do not influence cellulose binding of the enzyme (Simpson and Barras 1999).

It is interesting to note that aromatic residues may also play a significant role in the binding of CBMs to noncellulose polysaccharides. Mutants Y19A and Y85A in

the N1 CBM of Endoglucanase C from *C. fimi* reduce affinity for both phosphoric acid swollen Avicel and the soluble polymer barley β-glucan (Kormos et al. 2000). Furthermore, mutagenesis of solvent-exposed tryptophans W17, W54, and W72 of the family 2a carbohydrate-binding module (CBM2a) of xylanase Cel10A from *C. fimi* showed the aromatic nature of residues at these positions is important for binding to crystalline cellulose, yet residues with hydrogen bonding potential on the same face of the protein did not significantly influence binding (McLean et al. 2000). These reports indicate that aromatic residues are observed in many CBM/polysaccharide interfaces, which indicates that hydrophobic interactions may represent a conserved feature common to all types of CBM.

Recent studies have indicated that CBMs may bind to more than one polysaccharide. For example, X-ray crystallography and mutagenesis have shown that the family 11 CBM (CtCBM11) from *C. thermocellum* Lic26A-Cel5E has a common binding site for a range of β-1,4- and β-1,3-1,4-mixed ligands (Carvalho et al. 2004). Furthermore, the CBM29-2 from *Piromyces equi* NCP-1 cellulase/hemicellulase complex binds cellulose, mannan, and glucomannan (Flint et al. 2004), whereas the E78R mutant of retains significant affinity only for cellulose (Flint et al. 2004), demonstrating that the specificity of CBMs is a property that may be modulated by engineering the polysaccharide-binding site. A more complete understanding of the topological and physico-chemical factors that determine CBM affinity for a given polysaccharide would open novel possibilities for biotechnological application for engineered CMBs (Shoseyov et al. 2006).

4.4 Cellulose (Substrate) Binding to the Catalytic Domain

Cellulolytic enzymes can be broadly categorized as either endo- or exoglucanases, and the mode of cellulose binding to the different enzymes plays a major role in determining the catalytic outcome. Endoglucanases hydrolyze internal β-1,4 glycosidic bonds in cellulose and must therefore bind long-chain polymers. The three-dimensional structures of endoglucanases of families 5 (Varrot et al. 2001), 6 (Larsson et al. 2005), 8 (Guerin et al. 2002), 9 (Mandelman et al. 2003), 12 (Sandgren et al. 2003), and 44 (Kitago et al. 2007) reveal that the active sites are located within surface clefts that include a series of adjacent glycoside-binding sites, and site-directed mutagenesis has confirmed many features of substrate binding predicted from the crystal structures (Barr et al. 1998; McCarter et al. 2002; Rignall et al. 2002; Li and Wilson 2008). Exoglucanases hydrolyze either the reducing or nonreducing ends of the cellulose polymers to release cellobiose and are characterized by deep extended surface clefts (e.g., families 3 (Sakon et al. 1996) and 9A (Schubot et al. 2004)) or tunnels (families 7 (Divne et al. 1998) and 48 (Guimaraes et al. 2002)) incorporating the active site. For example, the crystal structure of the Cel7A cellobiohydrolase from *T. reesei* (CBHI) indicates that the substrate-binding tunnel is lined by ten subsites, each capable of binding a β-1,4-linked glucopyranose (Divne et al. 1998).

The availability of three-dimensional structures permits application of rational design strategies to study and improve catalytic performance by engineering of the substrate-binding site. For example, the D232A mutant in the substrate-binding cleft of the Cel5 endoglucanase from *Macrophomina phaseolina* (Eg11) changes the requirement of a minimum of five glucosyl units to six (i.e., subsite engineering) (Wang and Jones 1997). In a separate study, the K259H mutant in glucosyl subsite −2 of *Thermobifida fusca* Cel6B increased activity 3.7-fold on carboxymethyl cellulose substrate, and H159S and N190A mutants in subsite +1 of the enzyme had 6.6- and 5.0-fold higher k_{cat}/K_m values against 2,4-dinitrophenyl-β-D-cellobioside, respectively (Zhang et al. 2000a).

Cellulase inhibition by the cellobiose product and limited catalytic processivity are properties that may impede the industrial scale application of these enzymes in biofuel production, and engineering the substrate-binding cleft to minimize these effects may represent a promising solution. In the case of *T. fusca* Cel6B, mutants E495D, H326A, and W329C in the glycosyl substrate subsites −2, +1, and +2 were found to significantly reduce cellobiose feedback inhibition of the enzyme (Zhang et al. 2000b). Engineering of binding site accessibility may lead to altered processing of the cellulose; for instance, the family 9 cellobiohydrolase from *C. thermocellum* (CbhA) is exclusively an exocellulase, rather than an endocellulase commonly found in this family. The crystal structure of the E795Q CbhA mutant in complex with cellotetraose shows that a surface loop occludes the −2 cellulose subsite, which impedes the binding of long cellulose chains explaining the exo- rather than endocellulase activity (Schubot et al. 2004). Understanding the structural determinants of endo- and exocellulase activities is the first step toward engineering improvements in the hydrolytic activity against recalcitrant crystalline substrates.

4.5 Engineering the Catalytic Mechanism of Cellulases

The chemical events in cellulose hydrolysis involve highly conserved pairs of anionic residues in the active site which act as a general acid (proton donor) and a nucleophile/base in the catalytic cycle (Davies and Henrissat 1995). These mechanisms are supported by a wealth of mutagenesis experiments, which have been the subject of an excellent review article (Schulein 2000). The focus here is on models of enzyme mechanism, which necessarily integrate concepts of substrate recognition, substrate binding, modification of substrate properties, and enzyme processivity both before and after the catalytic event.

A model for the processive action of the Cel48F endocellulase from *Clostridium cellulolyticum* has been proposed based on the crystal structures of enzyme/substrate and enzyme/inhibitor complexes. During catalysis, the cellulose substrate must slide through a series of subsites in a tunnel lined with aromatic residues. The association of sugar residues in the cellulose with tryptophan residues in the tunnel subsites is suggested to facilitate a sliding motion of the substrate in order to

continuously supply the leaving group site with sugar residues after the catalytic cleavage (Parsiegla et al. 2000). Subsequent crystallographic studies of complexes between thio-linked glucoside inhibitors and the inactive mutants E44Q and E55Q have led to the proposal of a two-step mechanism in which the processive action and cellulose chain disordering are controlled by occupancy of discrete subsites of the enzyme (Parsiegla et al. 2008). This model may be compared with computer simulations of the complex between the endo/exocellulase Cel9A from *T. fusca* and a cellulose 18-mer, which predicted residues involved in glycoside binding in the CBM and CDs. The proposed mutant F476Y was experimentally shown to improve activity by 40% against soluble CMC and amorphous swollen cellulose substrates (Escovar-Kousen et al. 2004). It is noteworthy that both the processive models of the *T. fusca* and *C. cellulolyticum* enzymes involve a sequence of subsites that disrupt the cellulose prior to guiding the polymer to the active site.

Protein flexibility is also important for cellulase function, and site-directed mutagenesis has suggested that loop motions may play a key role in regulating enzyme activity (Davies et al. 1995; Zhang and Wilson 1997). Double cysteine mutants created to cross-link surface loops in the active site cleft region of *T. fusca* endocellulase Cel6B decreased the catalytic activity, suggesting that movement of these loops is important (but not essential) for function of the enzyme (Zhang et al. 2000b). In a particularly clear study, mutagenesis demonstrated a catalytic role for Asp79 in the Cel6A (E2) from *T. fusca* (Wolfgang and Wilson 1999), yet on the basis of the crystal structure of an enzyme–substrate complex this residue is 13A from the scissile bond (Spezio et al. 1993). Molecular dynamics simulations identified a conformation of the loop containing the Asp79 which placed residues Tyr73, Arg78, and Asp79 in the active site. A model was suggested which includes a significant conformational shift in the protein upon substrate binding (Andre et al. 2003).

Site-directed mutagenesis in the active site region has been shown to be a viable strategy to overcome product inhibition in the endoglucanase Cel5A from *Acidothermus cellulolyticus* (Baker et al. 2005). In this study, analysis of the crystal structure identified Y245 as a key residue forming stacking interactions with the cellobiose product after catalysis. This interaction was lost in a Y245G mutant, resulting in a remarkable 1,480% increase in K_i for inhibition of the enzyme by cellobiose and a 40% increase in the release of soluble sugars on treatment of biomass cellulose with the mutant enzyme (Baker et al. 2005).

5 Enzyme Chimeras

5.1 CBM/Catalytic Domain Fusions

The previous sections have highlighted the diversity of CDs and CBMs that are encountered in nature. As bioprospection and genome-sequencing projects gather pace, an increased number of widely diverse domains are likely to be discovered,

creating opportunities for creating chimeric enzymes in combination with CBM and CDs which target catalytically optimized enzymes to specific polysaccharides. The fusion of CBMs with CDs from different sources to form novel functional chimeras has emerged as an effective strategy to immobilize cellulase CDs on cellulose-rich substrates (Jamal-Talabani et al. 2004; Kim et al. 1998; Palonen et al. 2004). Perhaps as a consequence of their planar polysaccharide-binding surfaces, the Type A CBMs are relatively easily exchanged, and this facility has been exploited to create a novel enzyme with increased hydrolysis against crystalline cellulose (Mahadevan et al. 2008). Fusions of unrelated CBMs and CDs from widely differing sources have been created, such as that between a type C CBM (CBM49) from the tomato Cel8 (SlCel9C1) and the CD of the endoglucanase from the bacteria *T. fusca* (Urbanowicz et al. 2007).

5.2 Multifunctional Enzymes

Cellulolytic activity in many bacteria and fungi is achieved by the activity of the cellulosome, a large extracellular assembly comprising enzyme modules noncovalently linked to a membrane-bound scaffold protein, which has been the focus of many reviews (Doi 2008; Schwarz 2001; Felix and Ljungdahl 1993). The association of enzymes to form supramolecular assemblies may increase the overall catalytic efficiency of the complex in comparison with activities of the individual enzymes and provides novel possibilities for regulation of the enzymatic activities through the relative spatial positioning of catalytic sites (Conrado et al. 2008). During evolution, the genes encoding the individual enzymes may be fused to form discrete domains in a multifunctional enzyme, in which the different catalytic activities are manifested in a single polypeptide (Meier and Burkart 2009). Natural cellulase fusions include the natural chimeras Lic26A-Cel5E from *C. thermocellum* that contains family Cel5 and Cel26 domains with β-1,4- and β-1,3-1,4-endoglucanase activities (Carvalho et al. 2004), the Cel9D-Cel44A (previously called CelJ) from *C. thermocellum* (Ahsan et al. 1996), the CeAB from *Teredinibacter turnerae* that displays both a cellobiohydrolase and β-1,4-1,3 endoglucanase activities (Ekborg et al. 2007), and the bifunctional endoglucanase/endoxylanase from *Cellulomonas flavigena* (Perez-Avalos et al. 2008).

Hybrid enzymes may be created using recombinant DNA technology, recruiting catalytic functions from enzymes found in bioprospection programs, or enzymes created by protein engineering strategies as modules for combining catalytic activities not found in nature (Doi and Yanagawa 1999; Lu 2005; Nixon et al. 1998). The gene encoding the natural multifunctional enzyme cel44C-man26A was cloned from *Paenibacillus polymyxa* GS01 and the 1,352 residue protein encodes glycosyl hydrolase family 44 (GH44) and glycosyl hydrolase family 26 (GH26) CDs together with a fibronectin domain type 3 and a cellulose-binding module type 3, respectively. The mutants E91A and E222A abolished the cellulase and xylanase activities independently in the two separate CDs (Cho et al. 2006).

6 Perspectives

A general model for cellulase hydrolysis has been proposed which incorporates many of the structural features of the endo- and exocellulases considered in this section (Ting et al. 2009). The model analyses an idealized two-domain cellulase during the hydrolysis of crystalline cellulose, in which the relative motions of the CD and the CBM are coupled through a peptide linker. Both the length and stiffness of the linker modulate the cooperative action of the CD and CBM domains, and the maximum hydrolysis rate is coincident with a structural transition of the linker to an extended conformation which permits maximal variation in interdomain mobility (Ting et al. 2009). The model not only highlights the importance of studying the properties of the individual domains to an integrated understanding of cellulase function, but also indicates that a full understanding of higher level organization typically observed in enzyme complexes will require the development of more sophisticated models incorporating descriptions of catalytic synergy and inter-domain regulation of activity.

The increase in number and diversity of enzymes and multienzyme complexes that are observed in Nature gives a hint to the wide potential for their future application in biomass saccharification. The current expansion in the knowledge base together with a richer appreciation of the regulation of the individual catalytic activities in multi-enzyme complexes will permit the engineering of novel catalytic combinations for the specific biomass feedstocks that are favored in differing global geographic regions.

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Chapter 10
Genetic Improvement of Xylose Utilization by *Saccharomyces cerevisiae*

Gustavo H. Goldman

1 Introduction

It is estimated that capital costs associated with lignocellulosic ethanol are about US $4 per gallon and that these need to be reduced by more than half to be economically sustainable (Hahn-Hagerdal et al. 2006; Gray et al. 2006). Complete substrate utilization is one of the prerequisites to render lignocellulosic ethanol processes economically competitive. This means that all types of sugars in cellulose must be converted to ethanol, and that microorganisms must be obtained that efficiently perform this conversion under industrial conditions. Biomass is composed of cellulose (40–50%), hemicellulose (25–35%), and lignin (15–20%) (Ragauskas et al. 2006; Lin and Tanaka 2006). Glucose constitutes about 60% of the total sugars available in cellulosic biomaterial. Fermentation of the available sugars in cellulosic biomass presents a unique challenge because of the presence of other sugars such as xylose and arabinose (C5 sugars). The degree of branching and identity of the minor sugars in hemicelluloses tends to vary depending upon the type of plant. The conversion of biomass to useable energy is, however, not economical, unless hemicellulose is used in addition to cellulose. An ideal microorganism envisioned for use in biomass conversion should:

1. Produce ethanol with a high yield and specific productivity
2. Have a broad substrate range and high ethanol tolerance
3. Be tolerant to the inhibitors present in lignocellulosic hydrolysates

S. cerevisiae meets many of these criteria, but it is unable to effectively use xylose as a sole carbon source. This chapter aims to summarize the main advancements in the genetic improvement of xylose utilization by *S. cerevisiae*.

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2 Ethanol Production by Xylose-Fermenting Yeasts

Organisms that ferment the C5 sugars in cellulosic biomass can be divided into two subgroups, namely naturally occurring and genetically engineered microorganisms. The naturally occurring microorganisms include *Pichia stipitis*, *Candida shehatae*, and *Pachysolen tannophilus,* while genetically engineered organisms with C5 fermenting capabilities include *S. cerevisiae*, *Escherichia coli*, and *Zymomonas mobilis* (Jeffries 2006; Hahn-Hagerdal et al. 2007; Chu and Lee 2007). Figure 1 shows D-Xylose utilization pathway in bacteria and fungi. Two pathways are available for the conversion of pento-aldoses to xylulose: isomerization-based pathways and reduction/oxidation-based pathways. The conversion of D-xylose to D-xylulose goes by way of xylose isomerase (*xylA*). The XylA pathway is functional in a majority of prokaryotes (e.g., *E. coli*, *Streptomyces* sp), a few fungi (e.g., *Piromyces*), and plants (*Hordeum vulgare*, *Oryza sativa*, and *Arabidopsis thaliana*). In yeasts, filamentous fungi, and other eukaryotes, this proceeds via a two-step reduction and oxidation, which are mediated by the predominantly NADPH-dependent xylose reductase (XR: *XYL1*) followed by the NAD^+-dependent xylitol dehydrogenase (XDH: *XYL2*), respectively, with xylitol as the pathway intermediate. The inability of *S. cerevisiae* to grow on xylose has been attributed to: (i) inefficient xylose uptake, (ii) a redox imbalance generated in the first two steps of xylose metabolism, (iii) insufficient xylulose kinase (XK) activity, and (iv) an inefficient pentose phosphate pathway (PPP). However, it has been observed that D-xylulose, the keto isomer of xylose, can be fermented to ethanol by the yeast (Richard et al. 2000). Under anaerobic conditions, the different coenzyme specificities of XR and XDH produce a cofactor imbalance yielding a great accumulation of xylitol as a by-product and consequently reducing the ethanol yield (Pitkanen et al. 2003; Jin et al. 2004). Thus, engineering in *S. cerevisiae* for an efficient xylose metabolic pathway to

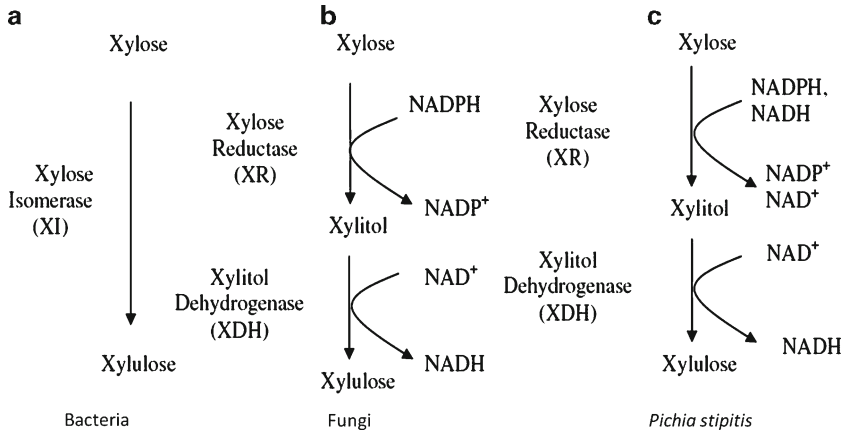

Fig. 1 Xylose utilization pathways by different microorganisms

produce xylulose can help to increase the overall yield and productivity of the fermentation process.

Whereas a large number of yeast species metabolize xylose and arabinose and display fermentative capacity, only approximately 1% of them are capable of fermenting xylose to ethanol. The requirement for electron acceptors translates to very low, carefully controlled, levels of oxygen required for maximum ethanol production from arabinose and xylose by these yeasts. However, such precise oxygenation is technically impossible to maintain in large-scale industrial conditions, with concomitant reduced product yield. The naturally pentose-fermenting yeasts are generally inhibited by industrial substrates and do not grow under anaerobic conditions even on hexose sugars.

P. stipitis is a haploid, homothalic, hemiascomycetous yeast that has the highest native capacity for xylose fermentation of any known microbe. Fed batch cultures of *P. stipitis* produce almost 50 g/l of ethanol from xylose with yields of 0.35–0.44 g/g xylose and they can ferment hydrolysates at 80% of the maximum theoretical capacity. *S. cerevisiae* regulates fermentation by sensing the presence of glucose, whereas *P. stipitis* induces fermentative activity in response to oxygen limitation. In yeasts, such as *S. cerevisiae*, ethanol is produced when sugar concentrations are relatively low, even under aerobic conditions (Crabtree effect). Unlike *S. cerevisiae*, *P. stipitis* is a respiratory yeast which does not produce ethanol under aerobic conditions, even in the presence of excess sugars. The choice to produce ethanol or cell mass by *P. stipitis* depends on the O_2 supply to the cells. At high aeration rates, only cell mass is produced, and at low aeration rates, ethanol is produced. *P. stipitis* is one of the few types of yeast that is able to ferment xylose to ethanol under anaerobic conditions because it possesses both NADH and NADPH-specific XR cofactors (Fig. 1). The benefit of XR using NADH is that there is a total cofactor balance when this cofactor is used, and therefore, no xylitol is produced. Kinetic studies indicate that NADPH is the preferred coenzyme because its affinity is about double the value for NADH under anaerobic conditions, and xylose fermentation by *P. stipitis* must proceed by NADH-linked XR for a total cofactor balance. Ethanol yields and productivity from xylose fermentation by *P. stipitis* are significantly lower than glucose fermentation by *S. cerevisiae* (Chu and Lee 2007).

3 Factors Limiting Xylose Metabolism in *S. cerevisiae*

The only, but major, inconvenience to use *S. cerevisiae* for lignocellulosic fermentation is its inability to metabolize and ferment the pentose sugars xylose and arabinose to ethanol. Although *S. cerevisiae* naturally harbors genes for xylose utilization, these are expressed at such low levels that they do not support growth on xylose. However, *S. cerevisiae* grows on D-xylulose and ferments it to ethanol, albeit with ten times lower rate than that for glucose. A recombinant *S. cerevisiae* strain expressing the *P. stipitis* XR (*XYL1*) gene is able to metabolize xylose, but

most of it is converted to xylitol (Amore et al. 1991; Hallborn et al. 1991; Takuma et al. 1991). Following this strain, another one was constructed by integrating both *P. stipitis* genes *XYL1* and *XYL2* (encoding *P. stipitis* XR and XDH) and the endogenous *XKS1* (encoding the *S. cerevisiae* XK). This last strain, named TMB3001, was the first one to demonstrate aerobic growth on 2% xylose as the sole carbon source (Eliasson et al. 2000), and it served as a starting point for subsequent xylose metabolic engineering experiments.

3.1 Xylose Uptake

Pentose uptake by yeasts requires transport proteins. Xylose is transported by the high- and intermediate-affinity hexose transporters in *S. cerevisiae* (Killian and Uden 1988; Meinander and Hahn-Hagerdal 1997). The affinity of hexose transporters for xylose is one to two orders of magnitude lower than the affinity for glucose and xylose uptake is competitively inhibited by glucose (Kotter and Ciriacy 1993). Previously, it was suggested that xylose is taken up by both high- and low-affinity systems of glucose transporters, but the uptake is increased in the presence of low glucose concentrations (Lee et al. 2002). Hxt4, Hxt5, Hxt7, and Gal2 are actually the most important transporters for xylose (see below; Hamacher et al. 2002) and a low glucose concentration is needed in the medium for efficient xylose uptake (Meinander et al. 1999). This can be possibly explained because the expression of glycolytic enzymes and the PPP need glucose (Boles et al. 1996; Meinander et al. 1999). In addition, it is also possible that glucose is needed for the expression of hexose transporters with favorable xylose transport properties, such as Hxt4 (Pitkanen et al. 2003; Bertilsson et al. 2008).

Therefore, in sugar mixtures, xylose is usually consumed only after depletion of hexose sugars. An active transport protein mechanism consumes 1 mol of ATP for each proton cotransported with the sugar to pump out the proton and prevent intracellular acidification. This functions under anaerobic conditions without creating major energetic constraints; however, the depletion of a significant fraction of the conserved ATP considerably reduces anaerobic growth. Therefore, better prospects for the development of pentose-fermenting *S. cerevisiae* are to consider heterologous expression of specific pentose-transporting facilitated diffusion proteins. *S. cerevisiae* strain TMB3201 was constructed using the same plasmid as strain TMB3001, but it has a disruption of 18 *HXT* genes and is unable to grow on 2% xylose (Wieczorke et al. 1999; Hamacher et al. 2002). Reintroduction and constitutive expression of individual *HXT* genes in strain TMB3201 revealed that at 2% xylose concentration, high- (Hxt7 and Gal2) and intermediate-affinity (Hxt4 and Hxt5) glucose transporters are required for xylose uptake (Hamacher et al. 2002). Interestingly, *S. cerevisiae* only expresses Hxt5 and Hxt7 when xylose is the sole carbon source (Diderich et al. 1999; Hamacher et al. 2002). Finally, Batista et al. (2004) have also identified the *AGT1* gene, encoding a high-affinity sucrose–proton symporter required for sucrose fermentation, as being also important for xylose transport.

3.2 Xylose Isomerase (XI)

Xylose isomerase catalyzes the isomerization of xylose to xylulose in bacteria and some anaerobic fungi (Jeffries and Jin 2000). Many attempts to express an active prokaryotic XI in *S. cerevisiae* have failed. These efforts were directed toward the XI expression from several sources, such as from *E. coli* (Sarthy et al. 1987), *Bacillus subtilis* (Amore et al. 1989), or *Clostridium thermosulfurogenes* (Moes et al. 1996), and none of them were active, suggesting that either yeast was unable to express *xylA* or to synthesize active enzyme. The first successful attempt was made with the *xylA* from the thermophilic bacterium *Thermus thermophilus* (Walfridsson et al. 1996), which was later significantly improved by mutagenesis, but it was not shown to be enough for efficient xylose conversion in yeast (Lonn et al. 2002). Kuyper et al. (2003) expressed with high enzymatic activity the *xylA* from the anaerobic fungus *Pyromyces* sp in *S. cerevisiae*. However, this enzyme was strongly inhibited by xylitol. Overexpression of the fungal *Orpinomyces xylA* gene is another successful instance for the anaerobic production of ethanol from xylose using recombinant *xylA*-expressing *S. cerevisiae* (Madhavan et al. 2009). Recently, Brat et al. (2009) screened nucleic acid databases for sequences encoding putative XIs. These authors were able to express in *S. cerevisiae* a new kind of XI from the anaerobic bacterium *Clostridium phytofermentans*. Although the activity and kinetic parameters of this new enzyme are comparable to those of the *Pyromyces* sp, the new enzyme is much less inhibited by xylitol, which accumulates as a side product during xylose fermentation. Furthermore, expression of the gene was improved by adapting its codon usage to that of the highly expressed glycolytic genes of *S. cerevisiae*.

3.3 Redox Imbalance from Xylose Reductase and Xylitol Dehydrogenase and Xylulokinase

Once inside the yeast cell, xylose is converted by a two-step oxidoreductive isomerization to xylulose (Fig. 1). Xylose is first reduced to xylitol by a NADH- or NADPH-dependent xylose (aldose) reductase (EC 1.1.1.21), and then xylitol is oxidized to xylulose by NAD-dependent xylitol dehydrogenase, a Zn^{+2}-dependent medium-chain alcohol/sorbitol dehydrogenase (EC 1.1.1.14). Xylulose is phosphorylated at the C5–OH position by Xylulokinase (XK) yielding xylulose-5-phosphate (X5P), which is further channeled into glycolytic intermediates such as glyceraldehyde-3-phosphate (GA3P) and fructose-6-phosphate (F6P) via the PPP. These intermediates are converted to pyruvate in the Embdem–Meyerhof–Parnas pathway (Nelson and Cox 2008). Under anaerobic conditions, pyruvate is decarboxylated by pyruvate decarboxylase to acetaldehyde which is then reduced to ethanol by alcohol dehydrogenase. As mentioned above, another alternative pathway for the conversion

of xylose to xylulose involves the use of XI by bacteria and some anaerobic fungi, but not yeasts (see previous section). The specificity of XR for NADPH and XDH for NAD leads to a redox imbalance that has been extensively studied in native xylose-fermenting yeasts (for a review, see Chu and Lee 2007). Yeast xylose metabolism is considerably affected by the availability of oxygen: aerobic conditions support biomass formation, while anaerobic conditions favor ethanol production. The redox balance of the two cofactor systems, NADH/NAD and NADPH/NADP, is importantly related to the aeration. During xylose aerobic growth of xylose-utilizing yeasts, formation and degradation of both cofactor systems are balanced, thus avoiding depletion of one of the nucleotide pools (Nissen et al. 2001). On the other hand, during anaerobic growth, xylose fermentation occurs, but it is normally followed by a redox imbalance of the NADH/NAD and NADPH/NADP cofactor pools. The redox imbalance occurs in yeasts that have XR and XDH reactions that are specific for the cofactors NADPH and NAD, respectively. Since these two enzymes have different cofactor specificities, NADP and NADH will accumulate during those oxireduction reactions. The accumulation of NADP can be minimized by regeneration of NADH under aerobic conditions via fructose-6-phosphate in the PPP and NADP-dependent enzymes isocitrate dehydrogenase and aldehyde dehydrogenase (Nelson and Cox 2008). In contrast, NAD is not recycled in sufficient amounts for the XDH reaction, what results in a great quantity of NADPH relative to NAD for the XR and XDR catalyzed reactions, respectively. Under conditions of reduced respiration, this cofactor-recycling imbalance results in inhibition of XDH activity and consequently increased xylitol formation and decreased ethanol production (Chu and Lee 2007). Karhumaa et al. (2007) have studied the xylose fermentation performance of a *S. cerevisiae* strain TMB305 that has high XR and XDH activity, overexpressed nonoxidative PPP, and deletion of the aldose reductase gene GRE3. The fermentation performance of TMB3057 was significantly improved by increased ethanol production and reduced xylitol formation compared with the reference strain TMB3001.

The excretion of xylitol has been mainly assigned to the difference in coenzyme specificities between XR (with NADPH) and XDH (with NAD^+), which creates an intracellular redox imbalance. XR has a higher affinity for NADPH than for NADH, whereas XDH uses only NAD^+, which leads to an excess accumulation of NADH and a shortage of NAD^+ necessary for the XDH reaction that cannot be recycled (Matsushika et al. 2008). *P. stipitis* XR differs from *S. cerevisiae* XR because it has dual cofactor specificity for NADPH and NADH. Accordingly, to change the coenzyme specificity of XR and/or XDH by protein engineering is an attractive approach for achieving efficient ethanol fermentation from xylose by using recombinant *S. cerevisiae*. Jeppsson et al. (2006) reported an enhanced ethanol yield combined with a decreased xylitol secretion in recombinant *S. cerevisiae* carrying the K270M *P. stipitis* XR mutant (that has a higher K_M for NADPH than the wild-type enzyme) together with the *P. stipitis* XDH wild-type. Watanabe et al. (2007a, b) constructed recombinant *S. cerevisiae* strains expressing the *P. stipitis* XDH and NADH-dependent *P. stipitis* XR. By using site-directed mutagenesis, the cofactor specificity of *P. stipitis* XR was changed from favoring NADPH to NADH aiming to help improving the intracellular cofactor redox imbalance. The recombinant *S. cerevisiae*

strains expressing the mutated NADH-preferring *P. stipitis* XR were able to ferment xylose to ethanol with an ethanol yield of 0.43 g/g when grown on a xylose (1.5%)–glucose (0.5%) mixture.

On the other hand, Watanabe et al. (2005) generated several *P. stipitis* mutants with complete reversal of coenzyme specificity toward $NADP^+$ by multiple site-directed mutagenesis on the amino acids from the coenzyme-binding domain and increased the thermostability by introducing a zinc-binding site. A quadruple mutant (D207A/I208R/F20S/N211R) showed more than 4,500-fold higher values in k_{cat}/K_M with $NADP^+$ than the wild-type enzyme, reaching values comparable with the k_{cat}/K_M with NAD^+ of the wild-type enzyme. They also constructed recombinant yeasts coexpressing NADPH-preferring *P. stipitis* XR and $NADP^+$-dependent *P. stipitis* XDH, and the resultant recombinant yeasts showed enhanced ethanol production followed by decreased xylitol secretion, probably keeping the intracellular redox balance (Watanabe et al. 2007b).

3.4 The Nonoxidative PPP Enzymes: Transaldolase and Transketolase

Two enzymes of the nonoxidative PPP, transaldolase (*TAL)* and transketolase (*TKL*), have been connected as being rate-limiting for xylose and xylulose fermentation. *TKL* catalyzes the conversion of X5P and ribose-5-phosphate to sedoheptulose-7-phosphate and GA3P, and *TAL* catalyzes the conversion of sedoheptulose-7-phosphate and GA3P to F6P and erythrose-4-phosphate. Glycolysis and PPP share GA3P and F6P, and accordingly, reduction of these compounds may contribute to inefficient xylulose fermentation. There are two main evidences supporting the involvement of PPP in this phenotype: (i) significant accumulation of the PPP intermediate sedoheptulose-7-phosphate during xylulose fermentation (Senac and Hahn-Hagerdal 1990) and (ii) elevated levels of *TAL* and *TKL* gene expression in *S. cerevisiae* grown in the presence of xylose/glucose (Toivari et al. 2004). Interestingly, overexpression of *TAL*, but not *TKL*, in recombinant *S. cerevisiae* expressing *P. stipitis XR* and *XDH* can improve the growth on xylose (Walfridsson et al. 1995). These results suggested that endogenous *TAL* activity is not sufficient to support the flux of xylose metabolites through the PPP. A recombinant *S. cerevisiae* strain (TMB3057) with improved growth on 5% xylose was constructed by expressing *P. stipitis XR* and *XDH* in a multicopy plasmid and an aldose reductase (*GRE3*) deletion (Karkhumaa et al. 2005).

4 Adaptation of *S. cerevisiae* Strains for Efficient Xylose Metabolism

Attfield and Bell (2006) applied natural selection and breeding over an extended period and developed *S. cerevisiae* strains that can double in less than 6 h using xylose as a sole carbon source. Strains with improved growth rate possessed

increased xylose reductase and xylitol dehydrogenase activities, with the latter showing the greater improvement. There are several instances where recombinant *S. cerevisiae* strains had their growth performance improved on xylose by adaptation to media where xylose was the single carbon source (for reviews, see Chu and Lee 2007; Hahn-Hagerdal et al. 2007). Unfortunately, in most of these cases the genetic modifications that precede adaptations have not been investigated in detail.

5 Concluding Remarks

The screening strategies for mutants or recombinant *S. cerevisiae* strains that have better utilization of xylose are far from being saturated. Several new approaches, such as fine-tuning expression levels of pathways components (synthetic promoter libraries and engineering posttranscriptional controls) and global perturbations for phenotypic improvement (random knockout and overexpression libraries, ribosome engineering, and targeting transcription/translation for introducing global perturbations), are just starting to be applied for genetic improvement of xylose utilization by *S. cerevisiae* (for reviews about these strategies, see Santos and Stephanopoulos 2008; Nevoigt 2008). Recently, global transcription machinery engineering (gTME), an approach for reprogramming gene transcription to elicit cellular phenotypes important for technological applications, was applied to improve xylose fermentation by *S. cerevisiae* (Liu et al. 2010). Mutation of the transcription factor *spt15* was introduced by error-prone PCR and then screened on media using xylose as the sole carbon source. Although modest growth rates were obtained in media containing xylose, the results were promising and demonstrated that the application of these new technologies can revolutionize this area.

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Part III
Plant Cell Wall Genetics

Chapter 11
Tropical Maize: Exploiting Maize Genetic Diversity to Develop a Novel Annual Crop for Lignocellulosic Biomass and Sugar Production

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1 Introduction

Maize (*Zea mays* L.) is truly a remarkable crop species, having been adapted from its tropical origins to a wide diversity of environments and end uses. According to the Food and Agriculture Organization of the United Nations FAOSTAT webpage, 792 million metric tons of maize were produced worldwide in 2007, making it the world's highest yielding grain crop (http://faostat.fao.org/site/339/default.aspx). When maize varieties adapted to tropical latitudes are grown in temperate environments such as the US Corn Belt, they flower later and produce little or no grain, but have higher total biomass yields compared to modern commercial corn grain hybrids (Fig. 1). Further, tropical maize also accumulates high amounts of extractable stalk sugar (sucrose, glucose, and fructose) because of reduced grain formation. Although offering potential benefits as a feedstock for biofuels, the direct use of tropical maize germplasm in temperate environments is hampered by greater lodging, less stress tolerance, and susceptibility to disease and insect pests – traits that have been greatly improved in modern US corn grain hybrids. However, hybrids derived from crossing temperate-adapted and tropical parents successfully combine the high biomass potential of tropical maize with the genetic improvements from the past century of corn breeding for high grain yields in temperate environments. Named "tropical maize," these tropical x temperate hybrids produce greater biomass and sugar compared to current US corn hybrids using at least 50% less nitrogen (N) fertilizer inputs (Table 1).

Tropical maize combines the wide geographic range, opportunities for genetic germplasm improvements, and established production practices for the maize crop with some of the positive environmental benefits and higher potential biomass

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Fig. 1 Picture taken on 26 Oct 2008 of a representative tropical maize hybrid (foreground) compared to typical commercial corn grain hybrids

Table 1 Yields of biomass, grain, and sugar for tropical and grain maize hybrids grown either with (225 kg N/ha) or without supplemental N at Urbana, Illinois in 2007

	Grain hybrids		Tropical hybrids	
	−N	+N	−N	+N
Yield component	Mg/ha			
Biomass	14.8	19.5	20	22
Grain	6.0	10.8	1.3	2.5
Sugar	0.6	ND	3.0	3.6

Average of 15 commercial grain hybrids or 7 tropical hybrids
ND not detected

yields attributed to perennial grasses. Tropical maize also offers efficiency gains in the direct conversion of sugar to biofuels and biobased products, potentially enabling the success of the Brazilian sugarcane ethanol–electricity cogeneration model to be applied in the US.

2 Advantages of Tropical Maize as Compared with Other Biofuel Crops

Issues of global climate change, national energy security, and boosting rural economies provide incentives to produce ethanol and other fuels from biobased feedstocks that include cellulosic plant materials. Converting a portion of the world's fuel supply from petroleum-based products will require an inexpensive, uniform, and renewable source of biomass, of which agricultural sources are expected to provide a substantial amount. No single feedstock type or land management practice will work for all locations. A joint 2009 workshop report of the US Department of Agriculture (USDA) and Department of Energy (http://genomicsgtl.energy.gov/biofuels/sustainability/sustainability2008_web.pdf) concluded that to understand the kinds of feedstocks and management regimes best suited for different landscapes, it is necessary to envision the whole system from production, management, and processing to ecosystem services, and from economic outputs to infrastructure and resource requirements for different feedstocks.

Determining the traits or characteristics needed for "a good biomass crop" can be a challenge due to the diversity of environments and the varieties of feedstocks under consideration. Further complications include economic factors related to costs of planting, harvest and transportation, yield per acre, fuel conversion efficiency (fuel yield), infrastructure for all parts of the crop life cycle, and potential social barriers if growers are reluctant to take the risk of growing a "new crop." Desirable attributes associated with biofuel crops include, but are not limited to, genetic resources for improvement, lack of invasiveness, latitudinal range of adaptability, availability of production and harvest equipment, and grower acceptance (Table 2).

A wide variety of plant species have been proposed as potential biomass crops to meet this need, each offering complementary advantages in different regions based on a number of important biological, economic, environmental, and societal factors. Among the many possible options for biomass crops, grass species that employ the C4 pathway for photosynthesis emerge as leading candidates because they exhibit the highest efficiencies of carbon fixation, water use, and nitrogen economy (Ragouskas et al. 2006).

Maize, sugarcane, sorghum, and switchgrass all belong to this group of C4 grasses and are already highly productive biomass crops that collectively are cultivated across the global spectrum of agricultural production environments. Depending on species, C4 grasses can produce one or more of three distinct types of biomass feedstocks: sugar, starch, and lignocellulosic biomass. Although different C4 grass crops may be specialized for one or more of these forms of harvested carbon, most also exhibit versatility in the types of carbon produced and thus can be tailored as appropriate feedstocks for specific environments or processing streams (Table 3). Corn grain is the major source of first-generation feedstock for ethanol, and corn stover with tropical maize, sorghum, sugarcane, and switchgrass the major biomass feedstocks in the US for second-generation bioethanol and other biofuels. Only sugarcane, sweet sorghum, and tropical maize exhibit the combination of sugar, starch, and lignocellulosic biomass. Tropical

Table 2 Comparison of biofuel feedstock crops and their associated attributes in relation to bioenergy production

Biomass attributes	Grain corn	Tropical maize	Perennial grass	Corn stover/ wheat straw	Short rotation woody crops	Sugar-cane
C4 photosynthesis	X	X	C3/C4	N/A		X
Low input		X	X			
Noninvasive	X	X	Varies	X	Varies	X
Winter standing		X	X	N/A	X	N/A
Easily harvested	X	X	X	Varies		X
High-yield density/acre	X	X			X	X
High water use efficiency	X	X		N/A		X
Drought tolerance		X	X	X		
Pest/disease resistance	X	X		X		
Use existing farm equipment	X	X		X		X
Fits in to annual current crop rotations	X	X		X		X
Produce on marginal land		X	X		X	
Widely adaptable	X	X	X	X	X	
Transferable technology available for immediate crop improvement	X	X		X		
Genetic resources and breeding	X	X				
Known technology for production	X	X		X	X	X
Grower acceptance	X	X		X	X	X
High stalk sugar content		X				X

maize is unique among the C4 energy grasses because of the extensive genetic resources for crop improvement. Furthermore, tropical maize can be produced throughout a broad latitudinal range including the Midwest and north central states of the US, where extensive corn grain production and a large number of ethanol production facilities already exist.

Unlike ethanol produced from corn grain, which has an energy balance ratio of 1.4–1.5 (i.e., energy invested vs. energy returned), the combined sugar and biomass of tropical maize would have an energy balance ratio approaching ten, similar to that of sugarcane or sweet sorghum (Goldemberg 2007). Thus, tropical maize provides more energy per unit of land area than corn grain ethanol and, as a result, is more efficient in land usage. An additional similarity with sorghum and sugarcane is the potential of tropical maize to reduce greenhouse gas emissions by combining two of the most effective feedstocks – sugar and lignocellulosic biomass. In processing the sugar, the biomass itself is cofired to provide most of the input energy required for distillation. In comparison to gasoline, ethanol made from cellulose and produced with power generated from biomass byproducts can result in an 86% reduction in greenhouse gas emissions (Wang et al. 2007). This release of greenhouse gas can be reduced still further if the CO_2 released from fermentation is recaptured photosynthetically into additional feedstock.

Table 3 Distinguishing features among C4 grass feedstocks for US renewable energy

Distinguishing features	Grain corn	Sugarcane	Sweet sorghum	Tropical maize	Corn stover	Switchgrass
Total annual biomass yields (~US average, dry Mg/ha)	8	25	20	20	7	10
Life cycle	Annual	Perennial	Annual	Annual	Annual	Perennial
Harvestable carbon form(s)	Starch	Sugar/lignocell	Sugar/starch/ lignocell	Sugar/starch/ lignocell	Lignocell	Lignocell
Feedstock stability	Years	Days	Days	Days	Months	Months
Genetic resources and breeding	Excellent	Limited	Limited	Excellent	Excellent	Limited
Drought tolerance	Moderate	Low	Good	Moderate	Moderate	Good
Cold tolerance	Good	Poor	Moderate	Good	Good	Moderate
Nutrient use efficiency	Low	Low	Moderate	Good	Low	Good
Potential for invasiveness	None	None	Low	None	None	Moderate
Alternative markets	Many	Table sugar	Molasses/forage	Sugar/forage	Forage	Forage
Commercialization status	Current	Current	In develop	In develop	In develop	In develop
Geographic range	Midwest/north central	TX, LA, HI	Midwest/ southeast	Midwest/north central	Midwest/north central	Midwest/ southeast
Harvest window	September–February	September–December	August–October	August–November	September–November	Fall
Fuel conversion efficiency	Moderate	High	High	High	Low	Low
Productivity on marginal lands	Low	Low	Low	Low	Low	Moderate

3 Tropical Maize as a Flexible Biofuel Feedstock

The United States is the largest user of energy in the world with the US Energy Information Administration reporting in the International Energy Outlook 2009 that the United States accounted for about 21% of total worldwide energy consumption in 2006 (http://www.eia.doe.gov/oiaf/ieo/world.html). Change in global energy use will require a change in US production and consumption of energy. The United States has access to significant amounts of biobased resources including those of the highly productive corn/soybean cropping system. This agro-ecosystem is still largely focused on providing raw materials for the food and feed industries rather than the biofuel industry. However, the increasing demand for "homegrown" biofuels and the desire to avoid drawing that biofuel from the food supply have created a market for new feedstocks to support the growing bioenergy industry. Tropical maize can help meet those goals, where it may be used as a biorefinery feedstock to produce ethanol and other useful biofuels from stalk sugars and lignocellulosic biomass as well as solid fuel for thermal energy recovery. Such dual purpose lignocellulosic/sugar feedstocks are receiving considerable interest for their versatility in carbon forms, as a temperate alternative to sugarcane and as an adjunct feedstock to corn grain-based ethanol production.

Highly efficient cellulosic ethanol production is still a few years away, and viable ethanol fermentations until then will focus on starches and simple sugars. Sugar can be processed for ethanol fermentation for about half the cost of starch (Jacobs 2006). Corn grain ethanol plants could be adapted quickly to ferment sugar from tropical maize. The remaining stover after sugar extraction could be burned for energy, used for cellulosic ethanol production, or potentially mixed with the dry distillers grains from grain-based ethanol plants to enhance feed value for ruminant animals.

Based on side by side comparisons of potential ethanol production from tropical maize hybrids and commercial grain hybrids, initial tests show that tropical maize has a theoretical yield of 25% more total ethanol per acre than commercial corn

Table 4 Comparison of partitioned and total potential ethanol yields of the three top tropical hybrids with no supplemental nitrogen to a top commercial hybrid grown without and with 225 kg/ha supplemental *N*

		Potential ethanolyield[a] (L/ha)			
Hybrid	*N* level (kg/ha)	Sugar	Stover	Grain	Total
Tropical 2	0	2,132	8,490	ND	10,622
Tropical 3	0	2,039	7,835	ND	9,874
Tropical 4	0	1,917	9,528	ND	11,444
Commercial hybrid	0	ND	3,647	3,301	6,947
Commercial hybrid	225	ND	4,170	5,040	9,210

ND not determined due to negligible amounts

[a]Estimates for ethanol production potentials, as set forth in the USDA report, The Economic Feasibility of Ethanol Production from Sugar in the United States (http://www.agmrc.org/media/cms/ethanol_fromsugar_july06_01FD7FEB35110.pdf), are 480 L/Mg of corn grain, 564 L/Mg sugar (sucrose), 471 L/Mg of stover

hybrids when all components of both crops (i.e., sugar, grain, and stover) are utilized for ethanol generation, and tropical maize does so with less N fertilizer (Table 4). Supplemental nitrogen was needed to enhance the potential ethanol production of grain hybrids (by 32%), largely to increase grain production, while a similar amount of fertilizer N had no effect on the already high potential ethanol production of tropical maize hybrids (Table 1).

4 Biological Properties Driving Superior Sugar Production and Biomass in Tropical Maize

Maize is classified as a "short-day" plant, meaning that the duration of the night must be longer than a critical threshold to trigger flowering. As a species that evolved in the tropics, maize flowers under approximately 12 h day/night cycles. Altering this photoperiod control to trigger flowering during the longer days and shorter nights of summer seasons in higher latitudes was a key factor in adaptation of maize to temperate environments like the US Corn Belt. Growing maize varieties adapted to the tropics in temperate environments results in several developmental changes associated with sensitivity to photoperiod. The rate of shoot maturation is slower, leading to more vegetative leaves, thicker stalks, and delayed flowering with disrupted coordination between production of pollen from the male tassel and emergence of the silks on female ears (King et al. 1972). Any seeds that are fertilized develop more slowly. Each of these environmental responses provides the benefit of prolonged photosynthesis and carbon fixation. When coupled with reductions in grain formation, the sugar from photosynthesis is not translocated to grain and converted to starch, but is instead retained in the stalk as sugar. By crossing temperate and tropical parental germplasm, the tropical maize hybrid achieves the high biomass of the tropical parent, while the temperate parent imparts improved agronomic traits, such as better disease and pest resistance, less lodging, abiotic stress tolerance, and plant architecture optimized for high-density planting (Below and Moose, unpublished results).

4.1 Tropical Maize as a Sugar Crop

The concept of exploiting maize for its potential sugar and ethanol production has a long history. Anthropologists suggest that a primary driver for the domestication of maize was the high sugar content from stalks of its wild ancestor teosinte, which may have had many uses including fermentation to alcohol for human consumption (Smalley and Blake 2003). The Aztecs made sugar from maize stalks long before the European discovery of the New World (Winton and Winton 1939). Throughout the past century, there are periodic reports evaluating maize as a potential sugar crop for alternative sources of table sugar (Clark 1913), alcohol

production (Gore 1947; Widstrom et al. 1984), or a higher value animal feed (Sayre et al. 1931; Singleton 1948; Campbell 1964; Marten and Westerberg 1972; Stake et al. 1973; Leshem and Wermke 1981). Each of these investigations showed that sugar concentrations and yields increased when grain production is minimized, either by severe drought stress, high plant population density, prevention of pollination by covering ears, or physical removal of the ear following pollination or genetic male sterility. However, these studies only considered temperate-adapted germplasm, and only one study (King et al. 1972) included a tropical line among the temperate-adapted varieties.

The stalk serves as a conduit for movement of sugars to developing seeds, and evaluations of maize as a sugar crop demonstrate that the stalk is also an alternate sink for sugars when photoassimilate production exceeds plant use. In addition to the rapid accumulation of sugars in the stalk, the link between assimilate production and utilization results in accelerated leaf senescence following removal of the earshoot or the prevention of seed formation in corn grain hybrids (Crafts-Brandner et al. 1984). Interestingly, the limited production of grain from tropical hybrids grown in temperate regions does not cause this accelerated leaf senescence; conversely, the plants remain green and retain sugars in their stalks until a killing frost. A combination of sucrose, glucose, and fructose begins to accumulate around the time of silk emergence, but the majority of stalk sugar is in the form of sucrose until frost damages cellular integrity and releases invertase, which can hydrolyze the available sucrose. This sucrose can be extracted from the stalk, similar to sugarcane, and readily fermented for ethanol production. Furthermore, by avoiding the energetic costs of converting sucrose into grain storage products such as protein and oil, more total carbon is available.

4.2 Tropical Maize as a Lignocellulosic Crop

The sugars extracted from tropical maize can be directed immediately to fermentation, but compared to corn stover crop residue from production of maize grain, tropical maize, at 20 dry metric tons per hectare, provides threefold more lignocellulosic biomass (Below and Moose, unpublished results). However, regardless of the source of biomass, for cellulosic ethanol production to be commercially viable, an understanding of the plant cellular structure and associated qualitative genetic factors are necessary to develop chemical and biological methods to overcome recalcitrance to recovery of sugars from cell wall polysaccharides. For this reason, alternative uses for the feedstocks, such as pyrolysis, direct catalytic conversion to energy-dense fuels, and even cofiring for electricity production, are recommended (Lange 2007). In this respect, gaining genetic control of cell wall formation and architecture is central to optimization of the lignocellulosic feedstock to its end use, be it for its abundant and easily hydrolyzed cellulose or other polysaccharides or its energy-dense lignin content.

4.2.1 Chemical Structure of Lignocellulosic Biomass

The fundamental principles of cell wall architecture are common to all plant species, but the kinds and proportions of the structural components can vary between cell wall types and in different species (McCann and Roberts 1991; Carpita and Gibeaut 1993). All plant cell walls contain cellulose microfibrils as their main scaffolding components, varying between 30 and 90% of the dry mass for different cell types. The cellulose microfibrils are tethered together with cross-linking glycans to form a network, which is embedded in a matrix of acidic polysaccharides. Plants make two distinct kinds of walls, those characteristic of grasses, like tropical maize, and those of dicotyledonous species, including woody crops (Fig. 2). In addition, most plants have mixtures of cells with primary walls and cells with thick, secondary, lignified walls.

A distinctive characteristic of grasses compared to dicots is that their primary walls are cross-linked with a hydroxycinnamic acid-rich phenylpropanoid network, similar to lignin (Carpita 1996). In primary walls of grasses, glucuronoarabinoxylans (GAXs) are cross-linked to themselves and around cellulose microfibrils by this

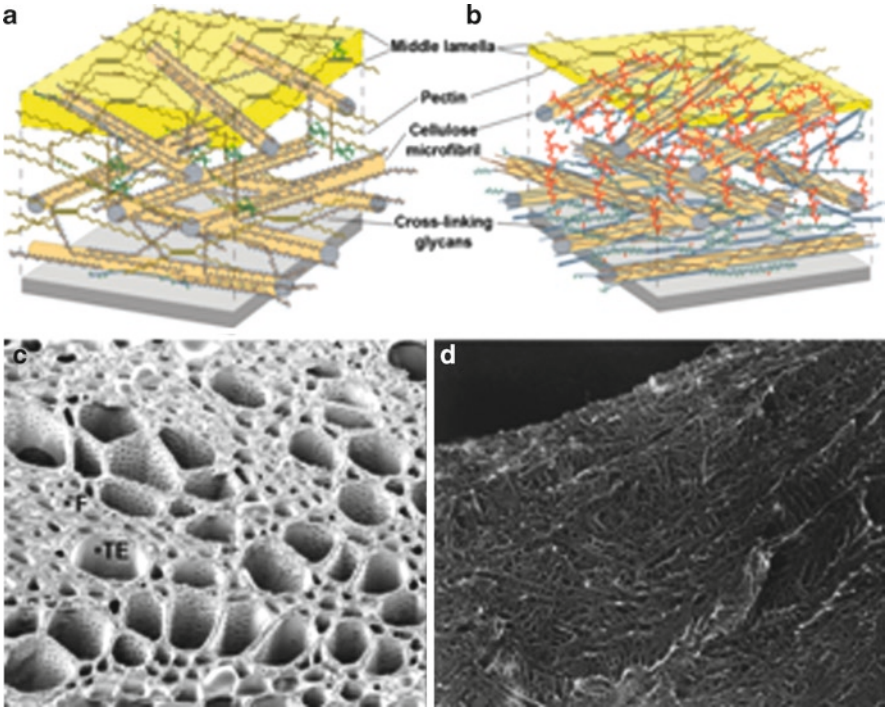

Fig. 2 Models of cell wall architecture in (**a**) primary walls of dicotyledonous species and (**b**) primary walls of grasses (Carpita and McCann 2008, adapted from McCann and Roberts 1991). (**c**) Scanning electron micrograph of secondary walls in xylem. (**d**) Transmission electron micrograph of maize epidermal wall molecular architecture. Reprinted from Carpita and McCann 2008, with permission from Trends in Plant Science

phenolic network. Hydroxycinnamic acids, such as ferulic acid and *p*-coumaric acid, are ester-linked to the arabinosyl units of GAX where they can serve as initiation sites for network polymerization. The lignin heteropolymer in the secondary walls is produced by coupling *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol subunits (collectively termed monolignols). The polymerization of these subunits leads to the formation of three types of lignin (*p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) lignin, respectively). H subunits are usually minor components, and the degree to which S and G units are incorporated into the polymer (commonly denoted as the S:G ratio) varies widely among species, tissue types, and even within an individual cell wall. More sophisticated methods of lignin analysis applied to a broader range of plant species, mutants, and transgenic lines have now revealed additional types of lignin subunits, including aldehydes, side-chain-reduced monolignols, and phenylpropanoid esters and amides (Lu and Ralph 1999; Boerjan et al. 2003).

4.2.2 Genetic Improvement of Lignocellulosic Biomass in Tropical Maize

Warm season perennial grasses, such as Miscanthus and switchgrass, are touted as prime candidates for low-input, sustainable energy crops, but they require specialized equipment and dedicated land space for production. As a large annual crop, tropical maize allows farmers to continue crop rotations and adjust their crops annually depending on markets. More importantly, maize is an outstanding genetic model species with the best potential for tailoring biomass for its end use (Carpita and McCann 2008). The tremendous, inherent, genetic diversity of all maize can be mined for genes impacting the downstream biological, thermal, or catalytic conversion of tropical maize. At present, only a handful of genes have been identified in grass species that contribute to biomass quantity or chemical composition. However, several thousand genes may affect these complex, quantitative, traits, controlling the ratio of primary to secondary-walled cells, cellulose crystallinity, cell wall composition and architecture, plant anatomy, or rates of cell growth. The tools of plant molecular biology and genetics enable the production of tropical maize plants in which these characteristics are optimized. For example, a critical aspect for the production of ethanol from digested lignocellulosic materials is to reduce the energy input required to break covalent and other linkages in the cell wall structure. Genetically altering lignin and the crystallinity of the microfibril structure in tropical maize can improve access of hydrolytic enzymes to their substrates (Ai et al. 2007; Himmel et al. 2007). Over 100 new cell wall mutants from a Uniform *Mu* population have been identified using both forward- and reverse-genetics approaches. In addition, a forward screen of field-grown lines using near-infrared (NIR) spectroscopic screen of mature leaves yielded several dozen lines with heritable, spectroscopic changes, indicating useful alterations in cell wall composition (Vermerris et al. 2007).

A knowledge base of the maize genes involving cell wall biology, expression profiles, and the phenotypic consequences of mutation is currently under development. The genome sequence of maize (Schnable et al. 2009) has resulted in over 750 maize

genes annotated and assembled into 21 gene families predicted to function in cell wall biogenesis (Penning et al. 2009). Comparative genomics and deep sequencing of transcripts in maize, rice, and Arabidopsis sequences have revealed differences in cell wall gene family structure and expression between grasses and dicots. Thus, development of tropical maize as a bioenergy crop will benefit directly from a grass-specific genetic model for functional analyses (Eveland et al. 2008; Penning et al. 2009).

Over a century of study of maize has provided new insights to unique mechanisms responsible for its rich genetic diversity. This diversity has been tapped to increase grain yields by over ninefold and should now be exploited for traits relevant to the improvement of tropical maize for both sugar and biomass production. Recombinant inbred lines, such as the Nested Association Mapping lines, which include several tropical maize germplasms, offer further sources of genetic diversity for identification of quantitative trait loci containing genes relevant for biomass improvement in a fraction of the time that was required for improvement of grain yield (Yu et al. 2008).

Biotechnology has also been a valuable means of improving maize and more biotechnology traits have been commercialized for maize than any other crop species. Through a combination of breeding and biotechnology approaches, genes may be identified and modified to enhance biomass and sugar production or provide benefits to the downstream saccharification and fermentation processes or other conversion routes. In contrast, biotechnology traits have yet to be commercially deployed in sorghum, partly due to concerns about gene flow from cultivated Sorghum to its close relative johnsongrass (*Sorghum halpense*), a pernicious weed (Morrell et al. 2005; Snow et al. 2005). In addition, the US biotechnology regulatory framework for dedicated perennial grasses crops such as switchgrass and Miscanthus is still being defined. While invasiveness is a concern for these perennial species in some areas of the US, no such concerns exist for maize.

5 Conclusion

Tropical maize represents an opportunity to grow more biomass on any of the estimated annual 90 million-plus US corn acres (http://www.ers.usda.gov/Briefing/corn/2009baseline.htm) with projected lower input costs than corn grain used for ethanol production. Near-term implementation of tropical maize is possible as it is derived from corn, a crop that has a well-established industry and infrastructure for support. Based on the genetic diversity and ample germplasm available, rapid advances can be made to genetically modify lignocellulosic and stalk sugar traits of tropical maize to meet current cellulosic ethanol processing and production requirements. Applicable crop production methods are well advanced and widely used, equipment is readily available, agricultural practices are sociologically ingrained in the producers, and a marketing, distribution, supply, and transportation infrastructure is already in place. Almost all aspects of the grain ethanol business model can be technologically transferred to tropical maize.

The unique crop characteristics of both sugar and biomass make the crop a ready supplement to current ethanol processes and a premier candidate for the long-range lignocellulosic production scheme. The significantly higher energy efficiency of tropical maize counters the argument that maize as a species is a poor choice as a bioenergy feedstock. Because tropical maize does not produce significant amounts of grain and requires less N inputs, it is on the periphery of the "food vs. fuel" debate and the associated questions about environmental sustainability that have been raised around the use of corn grain for ethanol. Tropical maize may also play a role in developing sustainable approaches to on-farm or community-scale energy production systems.

Due to the diversity and complexity of the burgeoning biomass industry, there is no panacea for our future energy requirements. Instead, a combination of existing mature biofuel technologies, including corn grain ethanol and soy-based biodiesel, along with second-generation ethanol feedstocks such as agricultural residues and third-generation feedstocks requiring research and development to be commercialized, such as perennial grasses, fast growing trees, and algae, will need to be used synergistically to formulate a sustainable energy future. The characteristics of tropical maize position it to be a high value stock in this energy portfolio serving as both a potential second- and a third-generation biofuel feedstock and promising to be an important component to the future energy economy.

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Chapter 12
Improving Efficiency of Cellulosic Fermentation via Genetic Engineering to Create "Smart Plants" for Biofuel Production

Zeyu Xin, Naohide Watanabe, and Eric Lam

1 Introduction

Biomass-based fuel is a near-term alternative to petroleum for powering the global economy in an ecologically sustainable fashion while minimizing the carbon footprint by decreasing net greenhouse gas emission. To affect this major shift in energy use and fuel source in the near future, dramatic improvement in the efficiency of converting cellulose into biofuels will be a key step. Optimization of downstream fermentation and separation processes through microbial engineering and industrial technologies integration are clearly important steps that need to be taken. In addition, altering feedstock properties to facilitate cellulose breakdown to energy-rich sugars will also play an important role in minimizing the environmental and financial costs for biofuel production. With the rapid advances in genomics and molecular techniques in the past decade, the stage is set for the design and engineering of candidate feedstocks to endow them with specific traits or transgenes in order to facilitate their disassembly. In this chapter, we will concisely review and discuss the considerations and present status on the use of genetic engineering as an approach to modify plants for optimal biofuel production.

1.1 Biofuels as Green Alternatives to Fossil Fuels: Considerations and Current Status

The advent of fossil fuel technologies in the past century has been instrumental in the rapid industrialization and growth of the global economy. However, the steady

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rise in documented ocean temperature in the past decades and dramatic permafrost melting at the polar regions in recent years provided strong evidence for large scale climate change as a likely by-product of fossil fuel consumption. On a per capita basis, the use of petroleum-based fuels alone generates about 2 metric tons of CO_2 annually worldwide. In more industrialized countries such as the United States, the per capita output reaches over 8 metric tons of CO_2 annually (http://www.eia.doe.gov/iea/carbon.html). Since CO_2 is only slowly returned to the earth after its release into the atmosphere, its concentration has risen steadily over the past century and much more rapidly in the past two decades, in pace with global industrialization and modernization. Its property as a greenhouse gas that can absorb and trap solar radiation, thus directly contribute to the climate change that is being manifested over the past decades. As countries in Asia and Africa become more industrialized in the coming decades, increased CO_2 output into the atmosphere and the heightened global demand for fossil fuels will likely hasten the rate of climate change as well as destabilize international relations. Thus, it is urgent that renewable and sustainable (i.e., "Green") energy sources should be found and deployed in the near future in order to retard or even reverse the adverse climatic effects due to fossil fuel use, in addition to providing more energy security internationally.

One of the alternative energy sources to replace fossil fuels is biofuel, which is produced from biomass. Compared to fossil fuels or renewable energy sources such as wind and hydropower, biofuel has an important advantage as the only fuel that is theoretically capable of being carbon-negative since plants can capture atmospheric CO_2 at the same time that solar energy is trapped by photosynthesis (Schiermeier et al. 2008). The production and consumption of biofuel appear to release only a portion of the carbon that plants captured from the atmosphere during their growth, thus the carbon cycle of biofuel usage can be neutral or negative. In addition, combustion of biofuels such as ethanol or biodiesel is known to produce less harmful gases than those generated from petroleum-derived gasoline and diesel fuels. Thus, the use of biofuels should have direct benefit to the air quality of large metropolitan areas such as Beijing and Los Angeles, as has been documented for Sao Paulo city in Brazil after the wide adoption of ethanol in replacing gasoline from their transportation grid.

The major first-generation biofuels include bioethanol, which is currently produced from either sugarcane or corn grain, and biodiesel which is mainly produced from oilseeds. Bioethanol has already made a significant contribution to global energy supplies. In 2007, the total global fuel ethanol production is about 13 billion gallons, about 40% of which is produced by Brazil from sugarcane. About 50% of the world fuel ethanol is from the United States (http://www.ethanolrfa.org/industry/statistics/) where it is primarily produced from corn grain. At present, bioethanol is the most common biofuel in the world. It can be used in petrol engines by blending with gasoline, commonly up to 10% (E10) on a volume basis. With Flex-fuel engines, vehicles can run on any ratio of gasoline and ethanol. Biodiesel is the most common biofuel in Europe and is available in many European countries. This oilseed-derived biofuel can be used in any diesel engine alone or by mixing with mineral diesel, and it provides almost the same energy per gallon as petroleum

diesel. Although biodiesel is widely used in the current transportation fuel system, total volume of production worldwide is comparatively low at present.

First-generation biofuels from available crop plants can be produced through technologically mature industrial processes. However, producing sugar-, starch-, and oilseeds-derived biofuels could potentially affect national food security in the future because sugarcane, cereal grains, and oilseeds by themselves are valuable food and feed commodity. As the demand and consumption of biofuels ramp-up, this pressure can be expected to result in competition between energy and food supplies if the present efficiency of biofuel production per area of farmland does not increase dramatically (Williams 2007). Thus, two key areas of biofuel production that urgently need to be improved are: (1) increase of total biomass output per hectare of available land, and (2) optimized conversion of various types of feedstocks into biofuels in order to maximize biomass conversion for logistical and economical reasons. For the first area, multiple energy crops adapted to different locales will likely be necessary to provide feedstock solutions to nearby refineries. Optimization of their production will likely require both accelerated breeding programs and mechanized farming practices. For this chapter, we will focus on discussing the prospect of using genetic engineering approaches to create feedstocks that will be more amenable to biofuel production by facilitating conversion of biomass components such as cellulose into fermentable sugars.

Second-generation biofuels will come from lignocellulosic biomass which includes cultivated feedstock and waste products such as corn stovers, rice straws, and wood chips. The use of agricultural waste and forestry residues means that the production of second-generation biofuels should not compete with the food supply for available farmland and natural resources. Moreover, the use of lignocellulosic biomass can potentially result in higher net energy gain than cereal grain or oilseeds if key technical problems can be resolved (Farrell et al. 2006). A key obstacle in cellulosic biofuel production is the complex components and structure of the cell wall in plants. Together with hemicellulose and lignin, cellulose fibers which are made up of long polymers of 6-carbon sugars, provide the strength and rigidity that is critical for the physiology and functions of plants (Rubin 2008). To release the energy-rich sugars from plant biomass, lignocellulose will need to be broken down via chemical, thermochemical, and/or enzymatic treatments. A typical scheme involving a combination of thermochemical and enzymatic steps to achieve cellulosic fermentation at the present time is depicted in Fig. 1. Currently, the price of cellulosic ethanol is about twofold higher than that of corn grain-derived ethanol or soybean-based biodiesel, due in part to the expense of chemical and physical pretreatments and to the cost for enzymes that are needed to convert lignocellulose into fermentable sugars (Ragauskas et al. 2006). The sustainable production of cellulosic biofuels will thus depend heavily on technological breakthroughs that improve processing efficiency and/or reduce the amount of energy/enzyme input, thereby lowering the cost. To help achieve this objective, one approach is to create more readily fermentable feedstocks through plant genetic engineering, which is the subject of this chapter. Feedstock optimization may be achieved through direct modification of the genome of energy crops to alter their cell wall composition, or

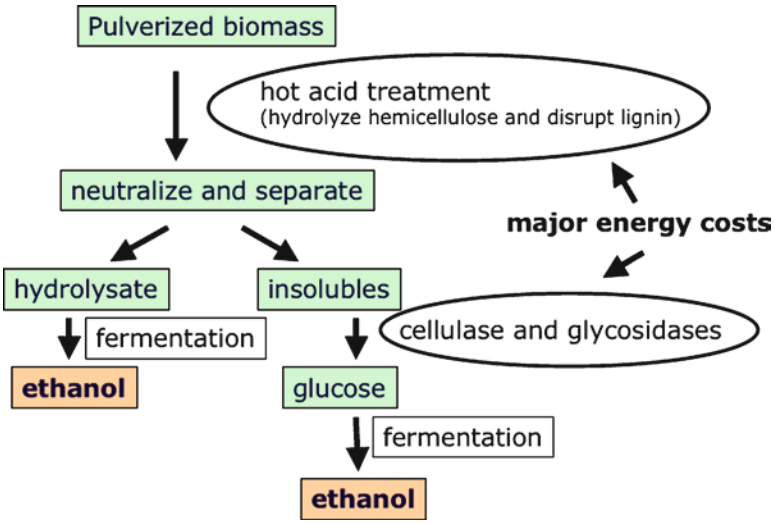

Fig. 1 Generalized scheme of cellulosic bioethanol processing. Current industrial process for cellulosic bioethanol production is depicted in a simplified scheme. Two points of major input in the form of heat, chemicals, and exogenous enzyme additions are identified. These inputs add a large deficit to the cost of fuel production from biomass and make the current price of cellulosic ethanol noncompetitive to bioethanol produced from sugarcane or even corn grain. If these costs can be successfully minimized, it will open the fuel market to the large amount of cellulosic biomass that can be produced worldwide at the present time. It should be noted that depending on the microbe being used for the fermentation steps, ethanol may be replaced with butanol or other types of biofuel as the final products

through overexpression of heterologous enzymes for lignocellulose degradation in transgenic plants. Subsequent processes of converting lignocellulose into fermentable sugars could become easier and more cost-effective using this type of genetically modified feedstocks specifically engineered for biofuel production – a kind of "Smart Plants."

1.2 General Strategies for Improving Biomass Deconstruction Efficiency Through Feedstock Alteration

Genetic engineering and plant breeding approaches are the main strategies to develop more readily fermentable energy crops. Traditionally, crop plants with desirable traits have been obtained through generations of successive selection after crossing different related species to maximize genetic diversity that have naturally evolved. A key to this approach is an efficient phenotyping strategy that allows for rapid and specific screens for the desired traits in large number of progeny from a segregating population. Coupled with methods such as somaclonal variation to generate novel genetic diversities and optimization of growth conditions to shorten generation

times, classical breeding have played major roles in the last "Green Revolution" to provide dramatic increases in crop production worldwide (Borlaug 1983). This approach will continue to be a major component of feedstock improvement programs in efforts to introduce stress tolerance traits and maximize yield production in target energy crops such as switchgrass and *Miscanthus*. In terms of biomass fermentability, however, major effort has predominantly been focused on improving sugar content of energy crops such as sugarcane, sweet sorghum, and sugar beets (Papini-Terzi et al. 2009; Sarath et al. 2008; Stich et al. 2008). More recently, cell wall components have also become targets for selective breeding programs of energy crops. For example, lignin level is a major factor that can affect the fermentable sugar yield in the pretreatment and hydrolysis steps during cellulosic fermentation (Chen and Dixon 2007). The average lignin content of mature switchgrass is about 17%, similar to that in maize stover and wheat straw (Carroll and Somerville 2009), with significantly higher levels in stem vs. leaf tissues. One may expect that dwarf species of switchgrass with increased leaf biomass contribution, thus a lower average content of lignin, can potentially provide an improved feedstock for more efficient biomass conversion. It is likely that availability of genomic tools in the relevant crop plants, such as the recently completed genome sequence of sorghum (Paterson et al. 2009), will provide a rich resource for molecular markers that will accelerate the breeding programs in various crop species (Karp and Shield 2008).

As an alternative to classical breeding approaches, advances in plant transformation techniques in the past two decades have enabled the directed genetic engineering of most plants through the production of transgenic plants. Harnessing the soil bacteria *Agrobacterium tumefaciens* as a gene transfer vector and the biolistic method for physical delivery of engineered DNA into plant cells, most plant species examined can be shown to be transformable to various extents. This ability to directly and specifically manipulate the genetic made-up of plants is now aided by the recent advances in genomic technologies that have made available a large repertoire of annotated genes from the completed sequence of many genomes (Rubin 2008). In the rest of this chapter, we will focus our discussion on how these new resources may be deployed to facilitate the rapid optimization of feedstocks to be more fermentable, thereby contributing to the development of sustainable biofuels.

2 Genetic Engineering Approaches to Improve Biomass Fermentability

2.1 Overexpressing Cell Wall-Degrading Enzymes in Plants: Tapping the Plant's Own Machinery for Deconstruction

Most of the cell wall degrading enzymes being used commercially originate from bacteria or fungi (discussed in section "Approaches to Enhance Protein Expression of Cell Wall-Degrading Enzymes in Transgenic Plants"). Currently, these enzymes

are mainly produced in microbial tank and thus generate significant carbon footprint during their production as well as substantially add to the net cost of biofuel production. In lignocellulosic fuel production, the necessary enzymes are applied to help deconstruct biomass and facilitate conversion of lignocellulose into fermentable sugars (Fig. 1). They are usually costly and the enzymatic hydrolysis efficiency is relatively low. Therefore, their preparation method needs to be improved so that cheaper and more effective enzymes can be obtained through better process designs and protein engineering. On the other hand, it has been recognized in recent years that plants can be used to produce cell wall-degrading enzymes themselves, thereby facilitating the dismantling of their own cell wall materials upon harvesting and pretreatment of the biomass prior to fermentation. By inserting into the plant's own genome the gene(s) encoding the necessary enzyme(s) to disassemble lignocellulose, one may be able to obviate or at least minimize the necessity of adding these components during the pretreatment steps. This kind of cellulosic feedstock would significantly decrease the cost of biofuel production and improve this technology's economic viability. Minimizing the need of adding commercial enzymes will also lower the net carbon footprint for production of cellulosic fuels and thus would be more ecologically friendly.

2.2 Tools for Engineering Cell Wall-Degrading Enzymes in Feedstocks

With rapid advances in DNA sequencing technologies and bioinformatic tools, large numbers of microbial genomes have now been deciphered, among which many are biomass degraders from various natural habitats (Rubin 2008). This growing collection of genomic information provides a rich tool kit from which cell wall degrading enzymes that have evolved to function under various conditions, such as low pH or high temperature, may be found. Thus, by examining the genome of acidophiles and thermophiles, cellulases and other enzymes that are required for efficient break-down of plant cell walls in conditions of acid pH and high temperature may be identified and their gene sequence could easily be cloned or synthesized. Using the approach of metagenomics and ultrahigh-throughput sequencing technologies, one can also begin to characterize complex communities such as the microbial flora in the termite hindgut (Warnecke et al. 2007) at the molecular level in order to better understand how cellulose in biomass can be efficiently processed in biological systems. In this regard, a more systematic characterization of the microbial and insect communities that have evolved to recycle biomass rapidly, as well as the temporal production of different enzymes for cellulose break-down in different context will definitely benefit our conceptual understanding of this process. For this purpose, locales such as tropical rain forest with rapid decay of fallen trees and shrubs should be interesting for exploration and study using various biochemical, molecular, and genomic techniques.

2.3 Approaches to Enhance Protein Expression of Cell Wall-Degrading Enzymes in Transgenic Plants

To use plants as bioreactors for effective production of cell wall-degrading enzymes, a number of technical hurdles would need to be overcome. Firstly, the enzymes would have to be produced at sufficiently high levels in the target feedstocks, thereby facilitating the conversion of most of the lignocellulose to the desired product. One estimate placed this at expression levels of about 10% total soluble protein (TSP) in the transgenic plant (Sticklen 2008). This can be achieved in part through the choice of the proper promoter to drive gene expression and by using codons optimized for the particular plant species. Significant differences in relative promoter strength as well as codon usage preferences are known between dicots and monocots (Wang and Roossinck 2006). In order to maximize protein expression levels in transgenic plants with heterologous genes such as those from fungi and bacteria, the codons of the transgene should be matched to the usage preferred in the recipient plant (Xue et al. 2003). This can be accomplished by using a synthetic gene approach, using the desired protein sequence as the template to design the appropriate coding sequence. One study suggested that optimizing just the first 40 codons of the transgene is sufficient for efficient protein expression in maize tissues (Hood et al. 2007). In addition, translational enhancer sequences from plant viruses may also be incorporated in the transcript to boost protein expression levels in transgenic plants (Dai et al. 2005; Oraby et al. 2007).

Another consideration is sequestration of the target enzymes in subcellular compartments. Since cell wall plays an important role in the integrity and rigidity of plant tissues, it would be prudent to keep the lignocellulose disassembling enzymes physically separated inside the cell until their release after harvesting the plants. In addition, targeting of transgenically expressed proteins to subcellular compartments have been reported to provide higher levels of accumulation of the target protein, in addition to more active enzymes due to improved folding of the translated peptides (Sticklen 2008). To effect proper targeting of the target protein in plant cells, several different type of subcellular targeting signals have been well described (Table 1). Most of these involve either N-terminal (plastid, mitochondrion, vacuole, apoplast, peroxisome target signal 2) or C-terminal (peroxisome target signal 1) targeting peptides that may or may not be cleaved during transit through the particular membrane system (Bassham and Raikhel 2000; Peters and Small 2001; Jurgens 2004; Baker and Sparkes 2005). For targeting to the endoplasmic reticulum (ER), a cleavable N-terminal presequence in addition to a C-terminal ER retention sequence are required (Bassham and Raikhel 2000). Lastly, targeting the nucleus of eukaryotes can be easily achieved by attaching a short basic polypeptide sequence to the coding sequence of the desired protein (Meisel and Lam 1996). The ER may be an especially excellent compartment for targeted accumulation of desirable enzymes in the plant cell since this subcellular location may provide more stability due to lower protease concentrations, and better solubility of expressed proteins from the presence of molecular chaperones (Schillberg et al. 2003).

Table 1 Organelle targeting signals in plants

Location	Type of targeting signal	Properties
Nucleus	Nuclear localization signal (NLS)	Short clusters of basic amino acids
Endoplasmic reticulum	Signal peptide	Cleavable N-terminal presequence
	ER retention signal	C-termini, H/KDEL motif
Plastid	Transit peptide	Usually cleavable N-terminal presequence
Mitochondrion	Presequence/Transit peptide	Usually cleavable N-terminal presequence
Peroxisome	Peroxisome targeting sequence 1 (PTS1)	C-termini, a conserved short motif
	Peroxisome targeting sequence 1 (PTS2)	Cleavable N-terminal presequence
Tonoplast/vacuole	Signal peptide	Cleavable N-terminal presequence
	Vacuolar sorting signals	Internal short sequence at near N-terminal
		C-termini, targeting to protein storage vacuole
Apoplast	Signal peptide	Cleavable N-terminal presequence

In the past years, most of the transgenic work on expressing cell wall-degrading enzymes in plants has been performed with bacterial cellulases and fungal xylanase. Using the celEM1 gene encoding a thermostable β-1,4-glucanase from the anaerobe *Clostridium thermocellum*, Abdeev et al. (2003) demonstrated that functional cellulase can be expressed and targeted to the apoplastic space of transgenic tobacco. However, these authors observed growth abnormalities in the resultant tobacco leaves and suggested that the auxin pathway in the transgenic plants may be altered through the activity of the expressed cellulase in planta.

In contrast, a gene coding the catalytic domain of the acid- and thermostable *Acidothermus cellulolyticus* 1,4-β-endoglucanase (E1) and targeting to the plant apoplast has been successfully expressed in Arabidopsis, tobacco, potato, rice, and maize plants (Dai et al. 2000; Sticklen 2006). The expressed enzyme shows high accumulation levels in plant tissues, with up to 20% TSP reported in Arabidopsis (Sticklen 2006), and manifests in vitro activity without obvious deleterious effects. In transgenic rice, the expressed E1 enzyme was demonstrated to be active in facilitating the conversion of cellulose in rice straw and corn stover into fermentable glucose (Oraby et al. 2007). In this work, transgenic E1 protein was found to accumulate up to 4.9% of TSP in the transgenic rice lines examined, with no obvious effects on plant growth. After treating rice straw and corn stover with the ammonia fiber explosion (AFEX) procedure, the transgenically expressed E1 enzyme from rice soluble extract was shown to be capable of converting cellulose

in plant biomass to glucose. This proof-of-concept work clearly demonstrated the feasibility of using transgenic plants as bioreactors for the production of the necessary enzymes to disassemble cell wall materials into fermentable sugars.

In an effort to optimize the expression of the holo-E1 enzyme in transgenic plants, Dai et al. (2005) carried out a systematic study comparing several different promoters, 5′ untranslated leader sequences, and five different subcellular targeting signals including four different plant transit peptides for ER, vacuole, apoplast, and plastid localization as well as that from the native E1 protein for secretion. By comparing a number of independently transformed tobacco lines, this comprehensive study concluded that the optimal construct for E1 expression comprised of the promoter from a tomato *RbcS* (small subunit of ribulose 1,5-bisphosphate carboxylase) combined with the 5′-untranslated region from Alfalfa Mosaic virus (AMV) *RNA4* and the apoplast targeting signal from the pathogenesis related gene *PR-S* of tobacco. In a more recent study involving transgenic maize (Hood et al. 2007), the E1 encoding gene as well as a fungal cellobiohydrolase-encoding gene (*CBH 1*) were tested with three different subcellular targeting signals for the vacuole, ER, and the cell wall. Using an embryo-preferred globulin-1 promoter to drive transcription and optimizing the first 40 codons for maize translation, high E1 expression can be obtained from the vacuole and ER-targeting constructs while low expression was observed for the 16 independent lines generated with the cell wall-targeting construct. In contrast, for *CBH 1*, no expression was observed for the vacuole-targeted construct. This observation suggests that depending on the particular target enzyme for overexpression, the subcellular compartment that is optimal may differ and this can only be determined empirically at this time. In any case, for both the *E1* and *CBH 1* genes, the highest seed expression levels of active enzymes reached over 15% TSP – a level acceptable for industrial scale-up. Successful expression of transgenic cellulase in developing barley grains has also been achieved, albeit at lower levels of about 1.5% TSP (Xue et al. 2003). One important thing to note from these studies is that no abnormalities were observed in the transgenic plant seed and embryo development even when very high levels of cellulase enzymes are expressed. In contrast, expression of a fungal laccase gene targeted to the cell wall of maize embryos resulted in seed browning and limited germination (Hood et al. 2003). Repeated breeding and selection through several generations after crossing with a high oil germplasm was necessary in this study to increase expression level and germination rate in the laccase expressing lines. Interestingly, transgenic expression of a fungal Manganese Peroxidase involved in lignin degradation caused lesion formation and early cell death in maize when constitutively expressed but not when specifically expressed in the developing seed (Clough et al. 2006). Thus, controlled expression of transgenes in addition to subcellular targeting may be preferred for optimal enzyme production while avoiding deleterious effects on plant development.

To further improve enzyme production levels in transgenic plants, simultaneous targeting of the desired protein to multiple subcellular compartments has also been attempted with encouraging results. In this case, a fungal xylanase was transformed into Arabidopsis with either an N-terminal plastid (Rubisco activase) or a C-terminal

peroxisome (SKL) targeting signal, or both signals (Hyunjong et al. 2006). Significant enhancement of up to twofold in the amount of transgenic protein being expressed was found with transgenic Arabidopsis plants expressing xylanase protein targeted into both plastids and peroxisomes. In this study, the highest level of the fungal xylanase accumulation in transgenic Arabidopsis lines that can be reached is 5% TSP (Hyunjong et al. 2006). Continued optimization of this approach to target the desired protein products into multiple subcellular compartments simultaneously should help to further increase the levels of target enzymes that can be reached in transgenic plants.

2.4 Contrasting Nuclear and Plastid Transformation Strategies: The Mix-Stock Approach

Most of the published work in transgenic expression of cell wall-degrading enzymes has been carried out using *Agrobacterium* as the transformation vector to insert the desired gene into the target plant genome. Compared to nuclear transformation, plastid transformation often results in much higher levels of foreign protein accumulation (De Cosa et al. 2001). While the usual observed levels of accumulation for foreign protein is up to ~1% TSP in nuclear transformants, the levels of transgene expression can routinely reach to 10% TSP in transplastomic plants. Recently, Oey et al. (2009) overexpressed a proteinaceous antibiotic in tobacco through plastid transformation and showed that the target protein accumulated to an extremely high level (>70% of TSP). Similarly, through plastid transformation, the expression levels of endoglucanases cel6A and cel6B from the thermophilic bacterium *Thermobifida fusca* ranged from 2 to 4% of TSP in tobacco transformants (Yu et al. 2007). With optimization of the region, 10–15 codons immediately following the start codon of *cel6A*, termed the downstream box (DB), stable leaf expression of the transgene can accumulate up to 10.7% TSP in young, mature, or old leaves (Gray et al. 2009). Furthermore, biochemical analyses of the expressed Cel6A enzymes from leaf extracts demonstrated that both the catalytic as well as cellulose-binding domains are folded properly and functional. These studies clearly demonstrated that using plastid genetic engineering is a viable alternative approach to nuclear transformation to produce cell wall-degrading enzymes in planta.

In addition to higher levels of protein accumulation, other advantages afforded by plastid transformation are shown in Table 2, in which the various advantages and disadvantages between nuclear and plastid transformation approaches are contrasted. In general, due to the high copy number per cell of the inserted transgene in the plastid genome, 2–4% of TSP in leaf tissues can be routinely achieved for most proteins while more specialized promoters and other strategies would be necessary to increase protein accumulation above 1% for nuclear transformants. In terms of protein stability, it is likely that the environment within the plastids will have less general proteases as compared to the cytosol. Perhaps the most important advantages for transplastomic plants vs. nuclear transformants are: (1) by using

Table 2 Comparison of plastid and nuclear transformation for plants

	Plastid transformation	Nuclear transformation
Protein expression levels	Higher (>20% of TSP is possible)	Lower (usually<1%)
Translated protein stability	Usually stable	Uncertain
Position effect	Seldom occurs	Often occurs
Transgene silencing	Seldom occurs or absent	Often occurs
Transgene escape	Seldom occurs because of maternal inheritance in most crop species	Possible
Polycistronic transcripts	Yes	No
Suitability of plants	Narrower range of species	Most species
Technical complexity	More laborious and time consuming	Easier
Transformation efficiency	Lower in most suitable plant species	Higher

homologous recombination as the means for gene insertion, position effects due to variations in transgene location are eliminated with plastid gene expression. In addition, with more defined insertion structure, gene silencing is not a problem for transgenes in the plastid genome. (2) Due to maternal inheritance of plastids in most crop species, transgene "escape" into the environment is of minimal concern in comparison to pollen escape from nuclear transformants. This factor could be of ecological importance to facilitate the approval and acceptance of the engineered crop plant for agricultural release. (3) It is apparent that to efficiently convert lignocellulosic biomass into fermentable sugars, multiple enzyme combinations would be necessary. This requirement for "gene stacking" could be done more efficiently with plastid transformation since polycistronic transcripts can be translated efficiently in this organelle in contrast to the case in the cytosolic ribosomes. Thus, operons containing multiple enzymes to optimize different steps in lignocellulose break-down can be engineered into the plastid genome relatively easily while multiple selection and crosses will need to be performed with nuclear transformants.

Despite the advantages that plastid transformation offers, there are some limitations at the present time for its deployment. One of the issues is that it is a more tedious procedure that involves longer selection time in tissue culture in order to produce homoplastomic transformants. Furthermore, although more plant species outside of the Solanaceae family have recently been reported to be amenable to plastid transformation (Table 3), it remains to be rather challenging for most crop plants, especially monocots such as the grasses that are the prime candidate for high biomass energy crops. Even among dicots, the most efficient species for transplastomic plant generation remain to be tobacco. Because of these limitations, we are currently exploring a novel approach, designed as Mix-Stock technology, as an alternative to direct feedstock engineering (Lam et al. 2009). Instead of designing and engineering individual feedstocks such as maize, switchgrass or *Miscanthus*, we will create transplastomic "enzyme-stock plants (ESPs)" that are essentially bioreactors that can be easily scaled-up for industrial production of multiple enzymes in lignocellulose or starch conversion to fermentable sugars. At the present time, our prime candidates for ESPs will be high biomass varieties of tobacco and potato since they are the most robust in plastid transformation technologies.

Table 3 Higher plant species reported so far for successful plastid transformation

Species	References	Notes
Dicotyledonous species		
Tobacco	Svab et al. (1990)	Efficient
Potato	Sidorov et al. (1999)	
Tomato	Ruf et al. (2001)	
Carrot	Kumar et al. (2004a)	
Cotton	Kumar et al. (2004b)	
Soybean	Dufourmantel et al. (2004)	
Lettuce	Lelivelt et al. (2005)	
Cauliflower	Nugent et al. (2006)	
Poplar	Okumura et al. (2006)	
Cabbage	Liu et al. (2007)	
Sugar beet	De Marchis et al. (2009)	
Arabidopsis	Sikdar et al. (1998)	Inefficient
oilseed rape	Hou et al. (2003)	Inefficient
Lesquerella fendleri	Skarjinskaia et al. (2003)	Inefficient
Monocotyledonous species		
Rice	Lee et al. (2006)	Heteroplasmic and unstable
Maize	Ahmadabadi et al. (2007)	Feasible

Once created, these ESPs with target enzymes stably expressed at levels of over 10% TSP can be mix-processed with a variety of feedstocks and obviate the need of exogenous enzymes addition as shown in Fig. 1. One can envision that either ESPs and feedstocks can be grown and harvested separately or in some cases, they may be grown side-by-side and harvested together to minimize added costs that may be incurred. A major benefit of this Mix-Stock approach is obviating the need of transforming and optimizing expression of the target enzymes in different feedstocks separately. Furthermore, by expressing the desired enzymes in potato tubers, we believe this could afford a longer shelf-life for storage and transport of the enzymes in an economical fashion. We have begun to test this novel approach in our laboratory and hope to analyze its feasibility and efficacy in the next couple of years.

2.5 Modifying the Lignin Content in Lignocellulose Through Genetic Engineering

In addition to using the plant's own biosynthetic capability to produce enzymes for biomass break-down and conversion to fermentable sugars, efforts have also started to redesign the very composition of the cell wall in target feedstocks in order to create more easily degraded cell walls. Lignin is an important contributor to the architecture of plant cells and is involved in protecting plants against pathogen and pest attack. During the process of biomass deconstruction, lignin mediated

cross-linking of hemicellulose to cellulose polymers limits the accessibility of enzymes to cellulose, therefore limits biomass digestibility. The rate of cellulose hydrolysis to sugars is inversely proportional to the lignin amount in biomass, thus showing that lignin content is a critical factor for the digestion efficiency of cellulose (Chen and Dixon 2007). Wheat straw and corn stover contain less lignin than sugarcane bagasse and poplar, implying that biomass from herbaceous crops should be easier for conversion than those of more woody crops.

To reduce the costs of cellulosic biomass degradation and increase the cell wall digestion efficiency, modulating lignin content via downregulation of lignin biosynthesis pathway enzymes could be one candidate approach. Although the complete lignin biosynthesis pathway in plants remains to be clarified, suppression of lignin content using antisense technology or RNA interference (RNAi) in stably transformed plants has been demonstrated. For example, transgenic poplar has been produced with reduced lignin accumulation using an antisense RNA approach. The lignin content was reduced from 20.6 to 12.8% by downregulation of 4-coumarate CoA ligase (4CL) while interestingly, the cellulose content concomitantly increased by 15% (Hu et al. 1999). In another report, Piquemal et al. (2002) showed decreased lignin content in transgenic maize using a maize caffeic acid *O*-methyltransferase (COMT) antisense gene construct while a more recent study showed that the lignin modification in alfalfa by downregulation of each of six lignin biosynthetic pathway enzymes could reduce or bypass the need of acid pretreatment in biomass deconstruction (Chen and Dixon 2007). In this latter study, the fermentable sugar yield in some of the transgenic lines is nearly twice as much as that in wild type plants after pretreatment with hot acid. Thus, the pretreatment process may be eliminated or at least minimized in the future as this strategy of decreasing lignin is optimized. In addition to lowering the cost of cellulosic biofuel production, reducing the pretreatment process should also increase the efficiency of subsequent microbial fermentation since inhibitory compounds such as furans, furfural, weak acids, in addition to phenolic compounds are known to be liberated during these treatments (Keating et al. 2006).

Although the feasibility of decreasing lignin content in plants has now been demonstrated with these studies via suppressed expression of lignin biosynthetic enzymes, an undesirable consequence in plant agronomic stature should be avoided. For example, partial reduction of lignin may induce semidwarf or dwarf phenotype in wheat and rice plants, although it is unlikely to affect the mechanical strength of plant tissues (Gressel 2008). In addition, careful assessment of the engineered plants in terms of crop yield under field conditions as well as their biotic and abiotic stress tolerance capacity would need to be carried out since the loss of substantial amount of lignin may compromise these stress defense systems, such as disease resistance pathways. Lastly, the various enzymes in the lignin biosynthesis pathways may differ between different energy crops and therefore, the consequence of their suppression will need to be determined empirically. Basic research in this regard would be necessary to eventually allow predictive engineering of various feedstock species in the near future. More in depth discussion of this aspect can be found in a recent review (Sticklen 2008).

3 Future Perspective

Although some important advances have been made in plant genetic engineering for biofuel production, we are just at the beginning of this phase of our efforts to optimize cellulosic fermentation. At present, the challenge is the development of an efficient way to improve the bioethanol production and decrease the costs of biomass deconstruction. For future deployment, ethanol is not necessarily the most optimal fuel that one can produce from biomass. Butanol as well as other higher octane fuel alternatives and valuable products that are currently created from petroleum may be produced from cellulosic biomass at an industrial scale. The current pretreatment processes need much input in energy, chemicals, and microbial enzymes, thus resulting in additional expense that makes this approach economically unsound. In this review, we have described recent efforts and ideas to try to minimize these inputs via plant genetic engineering to either utilize the plant's own cells as bioreactors to produce high quantities of the lignocellulose degrading enzymes or to create more fermentable feedstocks by suppressing lignin production. A recent study using RNAi strategy to downregulate expression of a glycosyltransferase involved in glucuronoxylan (GX) biosynthesis in poplar showed that wood of these RNAi plant lines can be more easily digested by cellulase (Lee et al. 2009). Since GX is an important component of hemicellulose, this work further demonstrated that transgenic approaches to modify various characteristics of the lignocellulose matrix in target feedstocks is a viable approach to make biomass more fermentable. Combined with the optimized expression of cell wall-degrading enzymes in either the feedstock of interest, or by supplying them in the Mix-Stock approach via engineered ESPs, the future looks bright that these combinations should provide significant contributions to make cellulosic biofuel production an economically viable option to further reduce global reliance on petroleum-based fuels. To realize this objective quickly, however, we believe that a close coordination in basic research to better understand lignocellulose biosynthesis pathways in various feedstock crops, improvement of transformation technologies for these same target species, and rapid assessment of field performance of the engineered plants would be critical.

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Chapter 13
Sugarcane Breeding and Selection for more Efficient Biomass Conversion in Cellulosic Ethanol

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1 Introduction

Breeding is the more appropriate way to improve productivity in sugarcane. Since the implantation of the "Proálcool" Program in Brazil, the sugarcane productivity in the last 30 years has increased in proportion of 761 kg/ha/year (1.5%). This productivity increment rate is similar to the values related in Brazil and world for other important economical crops. The constant ongoing rise of new cultivars has been considered the major factor contributing to this growth, although agronomic technologies have also been important contributions. Historical international examples of the success of this strategy have been the control of important diseases such as smut (*Ustilago scitaminea*), common rust (*Puccinia melanocephala*), Fiji disease (virus) sugarcane mosaic virus, leaf scald (*Xanthomonas albilineans*), red rot (*Glomerella ucumanensis*), as well as sugar content and, very importantly for Brazil, precocious sugar accumulation, that have expanded the harvesting period from 3 to 9 months in Brazil. Recently, a new disease named as orange rust (*Puccinia kuehnii*) became an important actual research target. Several important cultivars actually in the market are not resistant to this desease, but some of the RIDESA cultivars, such RB857515, the more cultivated, are resistant to the actual races of this pathogen. Breeding for disease resistance in Brazil has been successful for the majority of diseases, in order that it is not common to use fungicides in sugarcane crops. This recent disease resistance traits means that the germplasm used in our breeding research has enough genetic variability to obtain genetic improvements, as well as fiber content and composition, which will be discussed later in this chapter.

A spreading concern in important vegetative propagated crops such as sugarcane are the consequences of limited genetic diversity among cultivars. This

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fact results from a selection process that severely erodes the genetic variability created from recombination among selected and related parental clones and, additionally, narrow cytoplasmic variation on which *Saccharum* spp. hybrids are based, given the recurrent use of a small number of *S. officinarum* parental clones and *S. spontaneum*, and small contributions of *S. robustum* and *S. barberi*. This classical interpretation of narrow diversity in commercial hybrids contrasts with breeding results that despite that, continue extending the genetic gains on several fronts. Given the highly polyploidy nature of sugarcane, allele dosage and allele composition in any given genotype are likely to be important in determining phenotypic performance. The genetic behavior of polyploidy could generate additional variability by itself. One consequence of polyploidy is subfunctionalization, in which the ancestral expression profile becomes partitioned among duplicated genes (termed homoeologs), such that one copy is expressed in a subset of the aggregate ancestral expression space (cell lines, tissues, organs, or stage), whereas the other copy is expressed in the remaining portion (Adams et al. 2003). It is in this context that genomic changes often can take place, including chromosomal rearrangement and consequent changes in gene expression, like unequal expression of duplicated genes, and silencing of one copy, for example. Significant alteration in duplicated gene expression is fairly frequent even at the level of development and maturation of a single cell (Hovav et al. 2008). Mechanisms of variation of gene expression changes in polyploids include DNA methylation, histone modifications, and antisense RNA control of epigenetic changes have been demonstrated (reviewed by Adams 2007). Indirect evidence substantiates subfunctionalization as an important consequence of polyploidy for plant evolution and development (Lynch and Conery 2000; Mochida et al. 2004). These results suggest that genetic variability in polyploids, despite narrow genetic base, could quickly generate phenotipic plasticity.

New efforts of introgression of genes of wild relatives or related genes seem to be an essential step, not only for the improvement of productivity traits, but qualitative aspects are also needed to higher efficiency sugarcane biomass conversion in cellulosic ethanol. However, we still do not have evidence of exhaustion of genetic variability to reach these objectives, but this, however, does not decrease the importance of these introgression efforts.

2 Characterization and Use of Biodiversity in Sugarcane Breeding

Historical descriptions of cultivar improvement processes in sugarcane could be linked to two major steps: (1) discovery of the fertility in sugarcane, which was followed by intensive international exchange of germplasm; (2) the success of the breeding of POJ 2878, first notable example of the power of interspecific crosses, which resulted at the same time, in disease resistance, increase in production, and quality.

2.1 Interspecific Cross

When considering intraspecific crosses, the objective of hybridization in not to maximize the heterosis, but to average the character in focus obtained during the cross, which mainly depends on the genetic additive effects of the genitors. For characters with high heritance, it is very important to predict the additive genetic values. On the other hand, if there is low heritance for a character, divergent genitors must be used, and in this case, the objective is to improve heterosis and general combining ability (GCA), which would be the quantification of the genetic divergence relevant aspect that use the molecular markers in sugarcane could make a great contribution (Landell and Bressiani 2008).

Contemporary commercial sugarcane clones are mostly hybrids between *Saccharum* species. These hybrids were largely created from *Saccharum officinarum* L. ($2n = 80$), and the wild specie *S. spontaneum* L. ($2n = 40–126$). This modern material often bears aneupolid chromosssome numbers leading to chromosomal increases after some crosses, behavior similar to allopolyploids (James 2004). The strategy improves tillering and decreases the shooting height. Due to these improvements, it is actually possible in Brazil to create five harvests in one field, with an average productivity of 80.7 ton/ha, still far away from 320 ton/ha in a single crop, real productivity achieved in some occasions, where irrigation, better crop system and climate conditions were associated.

S. officinarum has a high sugar content, thick culms (3.5 cm or more), low fiber content, weak tillering and has a superficial and reduced radicular system. In other hand, *S. spontaneum* culms are very rich in fiber and have a well-developed system with good resistance to biotics and tolerance to abiotic stresses, but do not have significative levels of sucrose in culms and is a highly polymorphic species. Plants vary from short, bushy with no stalks, to large-stemmed clones over 5 m in height. Strong variation also occurs to stalk diameter and leaf width. More importantly, it is highly adaptable: clones are found under drought stress in deserts, waterlogged conditions, and saline conditions. It is found from up to 2,700 m sea level to near sea level. The progenies obtained from crosses between *S. spontaneum* and *S. officinarum* were successively backcrossed with *S. officinarum* in order to recover the majority of favorable genes to high sucrose levels in the recurrent parental, process named "nobilization" by Jewist in 1921, in Java. This process is in fact a form of introgression breeding, and it is described as the use of unreduced gametes in the introgression of *S. spontaneum* into *S. officinarum*. This is known as $2n+n$ chromosome transmission. Heinz (2007) considered that the most logical mechanism to explain this fact was the formation of reduced *S. officinarum* gametes that are stimulated to double in chromosome number when fertilized by gametes of *S. spontaneum* or of hybrids involving *S. spontaneum*. Sugarcane improvement has been very dependent upon use of unreduced gametes, yet the controlling mechanism is relatively unstudied. At RIDESA, we are able to make interspecific hybrids between the modern hybrids and *S. spontaneum*, *S. barberi* and *S. sinensis*. However, very low seed set and seed viability is obtained a large number of crosses are need to get viable seeds. Other Brazilian sugarcane breeding units like CTC and IAC. In Brazil, have made some

preliminary introgressions of *S. spontaneum*, (Bressiani et al. 2005), but in general, interspecific and intergeneric crosses did not have result in commercial clones and publications in our country.

Molecular cytogenetics (D'Hont et al. 1996; Piperidis and D'Hont 2001; Cuadrado et al. 2004) and genetic mapping studies (Grivet et al. 1996; Hoarau et al. 2001) have shown that modern cultivars typically have 70–80% of chromosomes entirely derived from *S. officinarum*, 10–20% from *S. spontaneum*.

2.2 Intergeneric Crosses

Further interest also exists for genes present in the "*Saccharum* complex" (Mukherjee 1957), which involves genera that contribute to the origin of sugarcane such as *Erianthus, Ripidium*, *Narenga, Sclerostachya and Miscanthus*. Chromosome contig in sintheny *Sacharum*, *Miscanthus*, *Narega* and *Sclerostachya*, has shown that these canes present with similar citogenetic traits. This was the only group in which the chromosome number $2n = 80$. Narenga and Sclerostachya have $2n = 30$, and *M. floridulus*, $2n = 38$.

Crosses between distant members of this taxonomic complex have been reported, for example, between *S. officinarum* and *S. durra* or *Bambusa arundinaceae* (bamboo) (Raghavan 1952; Rao et al. 1967) and with Sorghum (Thomas and Venkatraman 1930), where some trends to dwarfism and early flowering were observed. Raghavan (1954) attributed the ease of how sugarcane's interspecific crosses could occur to the high polyploidy. These characteristics could attest that the nonreduction in the chromosomes in the meiosis could allow the chromosomes pairs to pair among themselves, without any result, which, in significant aneuploidy, could turn the cross nonviable. Crosses between *Sacharum hybrids* with *Miscanthus japonicum* have also been successful (Li et al. 1951). Cytological characterization of the progeny confirms that fertilization occurred and that reduced gamete of *Sacharum* hybrid with an unreduced gamete of *M. japonicum* cross has occurred, with very small numbers of univalent chromosomes. At RIDESA we have also got viable seeds of crosses between modern sugarcane hybrids and *Miscanthus*, and this could be one way to allow cultivation of sugarcane in sub-temperate climates, that includes in developing world, significant parts of agricultural land in South America, Africa, Australia and India. *Sacharum* crosses with *Erianthus, Imperata* and *Zea* and *Narenga* have been reported (Janaki Ammal 1941), progeny have shown different levels of ploidia, and cytological observations describe the presence of trivalent and quadrivalent chromosomes. Janaki Ammal concluded that this wide interepecific *Sacharum* variation could be a result of some internal pairability of the chromosomes derived from the pollen pairs (autosyndesis). However, this hybridization with far distant species does not yet have added important traits to modern commercial cultivars.

Some evidence suggests that *S. spontaneum* and *Miscanthus* are more readily nobelized by *S. officinarum* than are *S. narenga* and *Erianthus* (Loh 1947). This evidence points out an important contribution of introgression of genes of *Miscanthus* in *S. officinarum*.

Currently, considerable interest is focused upon use of *Erianthus arundinaceus* (Retz.) Jeswiet. This is reflected in the number of recent papers on the use of molecular cytogenetic techniques to definitively diagnose the products of introgression breeding (Dhont et al. 1995; Besse et al. 1996, 1997).

3 Breeding of Sugarcane in Brazil

The major programs in sugarcane breeding in Brazil are:

1. RIDESA (www.ridesa.com.br) (Rede Interuniversitária de Desenvolvimento do Setor Sucroalcoleiro – *Academic Network for the Development of Sugar-Alcohol Sector*: previously named IAA-PLANALSUCAR (founded in 1977), which was defunct and assumed by a group of seven Brazilian univerisities that include 142 researchers involved in sugarcane research. These institutions supply the cultivars with the initials RB. The numbers of its seedlings evaluated are still increasing exponentially, and since 2008, about 2,100,000 seedlings from families originated from about 3,000 crosses were evaluated yearly. Since 1967, 67 commercial cultivars were produced and have been launched, part of that corresponded to the cultivated 57% of the total area in Brazil, which were almost completely financially supported by industry.
2. CTC (www.ctc.com.br) (Centro de Tecnologia Canavieira, "Center of Sugarcane Technology"), was funded after dissolution of the COPERSUCAR (varieties with the initials SP), actually supply the cultivars with the initials CTC. It is supported basically by industry, and its cultivars account for about 43% of the total area cultivated with cane in Brazil. This program evaluates about 3,000,000 seedlings annually. Created in 1979, CTC has produced nine commercial cultivars (60 varieties at all, considering COPERSUCAR varieties). This research unit includes more wide research fronts in sugarcane research than any other institution in Brazil.
3. IAC (http://www.iac.sp.gov.br/UniPesquisa/Cana/Cana.asp) (Instituto Agronômico de Campinas, "Agronomic Institute of Campinas"). This institute was the pioneer in sugarcane breeding in Brazil. Beginning in 1894, the first cultivar competition experiments started. This program is supported by the São Paulo State government and partially by some industries, and evaluates about 500,000 seedlings annually.

The significant cooperation between these different research institutes with interchange of materials between them are responsible for the construction of breeding net in the exponential phase of growth, offering farmers and industries the possibility of cultivating dozens of cultivars of different groups of maturation, increasing the stability against biotic and abiotic stresses. All together, these efforts constitute the larger breeding program in the world, despite the late development, as compared with other countries such as Australia, India, Java, and others who are early pioneers in sugarcane breeding.

Other recent private companies such as CanaVialis, created in 2003 by the Votorantim Holding Company and bought by Monsanto in 2008, still do not have launched varieties, something that is anticipated to occur in 2010. This company evaluates about

1,500,000 seedlings annually, and it is the leading Brazilian research unit in the development of molecular markers for sugarcane. The company announced that a single marker is enough to differentiate the major Brazilian cultivars, and that with four markers it is possible do discriminate about 1,500 genotypes of its germplasm bank.

The history of more important varieties and breeding efforts can be seen in Table 1 below.

Table 1 Brief history of Brazilian sugarcane breeding and the major cultivars cultivated during different periods. At the Federal University of Alagoas-UFAL/RIDESA, Maceio, AL, lat. 9°13′S, long. 35°50′W

Time Period	Origin/breeding program	More important cultivars
Until 1930	Imported cultivars	Caiana, Creoula, POJ, Co, CP
Since 1930	IAC (Program of the government of São Paulo State)	IAC48-65, IAC50-134, IAC51-205, IAC52-150IACSP93-3046, IACSP94-2101, IACSP94-2094, IACSP94-4004, IAC91-2195, IAC91-2218, IAC91-5155, IACSP93-6006, IAC86-2480, IAC82-2045, IAC82-3092, IAC86-2210, IAC87-3396
From 1930 to 1970	Agriculture Ministry (RJ State) Agriculture Ministry (PE State)	CB41-76, CB45-3, CB46-47, CB40-69 IANE46-182, IANE55-17, IANE55-33
From 1970 to 1990	IAA-PLANALSUCAR (now as RIDESA)	RB72454, RB721012, RB70141, RB70194, RB725147, RB725828, RB735275, RB711012, RB729359, RB739735, RB765418, RB785148, R735220, RB785750, RB806043, RB825356
From 1970 to 2003	COPERSUCAR (now as CTC)	SP70-1143, SP70-1284, SP71-1406, SP71-6163, SP79-1011, SP80-1842, SP803280, SP80-1816, SP81-3250, SP832847, SP86-42, SP87-365,
Since 1991	RIDESA – federal university network RIDESA: composed of the breeder teams of the universities UFAL, UFRPE, UFRRJ, UFG, UFV, UFSCar, UFPR, and recently, UFS, UFMT and UFPI	RB785750, RB806043, RB825336, RB835089, RB835486, RB75126, RB83102, RB83160,RB83594,RB835019, RB855156, RB855453,RB 813804,RB835054,RB8452 57, RB855035,RB855113, RB855536,RB867515, RB 855511,RB855463,RB8451 97,RB845210, RB855036, RB865230,RB928064,RB92579, RB 93509,RB931530,RB863129,RB943 365, RB872552,RB943538,RB93252 0,RB925211, RB935744, RB925345
Since 2003	CanaVialis (bought by Monsanto in 2008)	Planned CV for 2010
Since 2004	CTC (Centro de TEcnologia Canavieira)	CTC1–CTC15

RJ Rio de Janeiro State; *PE* Pernambuco

The actual set of cultivars allows the harvesting during 9 months; in autumn, winter, and spring. These different maturation classes of cultivars have a dramatic impact in the profitability of the sugar and alcohol production in Brazil, and were mainly due to breeding efforts and good cultivar cropping management.

4 Sugarcane Breeding: Flowering and Crossing

Flowering is a very important factor for breeding, but has a negative impact when production is the goal, because during flowering, vegetative growth stops and the sugar accumulated in culms is used to support the energetic expenses of flowering and seed production. Flower induction in sugarcane normally takes place when there is a slowing down of growth due to decrease in photoperiod. The problem of poor or variable flowering as limitation for breeding crosses has been related both in temperate, subtropical, and tropical regions. Limited flowering patterns, including variable and long periods for flowering (sometimes longer than 10 years), as reported by breeders in other regions of the world (Berding et al. 2004), which prevents planned cross pollination. As a consequence, limited parental clones could be used, and the most desired combinations are rarely possible.

Work in several countries has shown that low temperature was detrimental to flower initiation and development of spikelet fertility, particularly maleness (Moore 1985, 1987). The floral initiation process takes place when the nocturne period is at least 11 h and 30 min, and is reversible. The development of stamen and pollen are thermosensible, needing temperatures higher than 19°C, and in regions near the equator, flowering could occur twice a year for the same cultivar (Stevenson 1965). This variability introduces serious obstacles to breeding, and in fact suggests the importance of microclimate in flower induction. Crossing stations of several Brazilian research institutes are localized in Alagoas and Bahia humid state regions (between latitudes 13 and 9°S). Excellent performance in flower induction and fertility was continuously obtained at the RIDESA Crossing Facility, localized in the city of Murici, state of Alagoas (09°13′S; 35°50′W; 450 m above sea level, with an annual relative humidity of 93.2%, annual rainfall of 2,363 mm, average minimal temperature of 18.9°C, and maximum average temperature of 27.1°C.

RIDESA has an online monitoring system for flower development used by breeder teams spread in several Brazilian states. This system includes data such as weekly inspections of flowering for each clone in their germplasm bank. Because each cross is designed with antecedence of months, the crossing facilities researchers have two options: slow down or speed up the flowering of one or two of the parental of each cross. Figure 1 shows some basic aspects of sugarcane flowering.

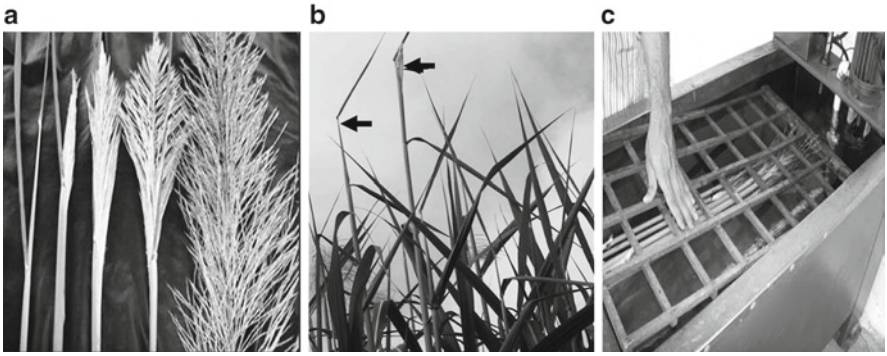

Fig. 1 Flowering evaluations and manipulations needed to perform a cross. (**a**) Visible and successive stages of flower development in sugarcane, (**b**) *arrows* indicate variability in flowering stages in a single ratoon: average values are needed to better describe differences between clones; (**c**) emasculation, typically done in biparental and multiparental cross, in order to verify that the fecundation pollen are not from female plants. Emasculation is also used when some degree of self-fecundation occurs in one progenitor

Knowing the flowering behavior in the field, flowering manipulation could be better designed. For example, increasing daylight hours to 12 h and 30 min could retard flowering as long as 53 days, as reported by James and Smith (1969). To speed up the flowering of one parental, they could be exposed to a shorter day. A database of flowering behavior is also already available for a large number of genotypes, and differences in induction time after photoperiod treatment could be previously analyzed in order to increase the success of the cross. Applications of excess of nitrogen could also delay the flowering. Several chemical products could be used to retard or prevent the flowering; an example of products that could retard flowering is ethephon (2-chloroethyl phosphonic acid), an ethylene-releasing compound, or herbicides such as maleic hydrazide, monuron, diuron diquat, and glyphosine (Moore 1985).

5 Common Crossing Strategies

Previous selected progenitors must be cultivated in order to flower in overlapped periods, preferentially, in order to get more seeds and better progenie evaluation. After testing the pollen viability, it is necessary to protect the inflorescence, isolating it from the others in an environment free of pollen from other sugarcane plants. Generally, the inflorescence is introduced inside a lantern made from cotton tissue. The two major cross methodologies used in sugarcane are the biparental cross and the cross pollination (Fig. 2).

Fig. 2 Major crossing strategies used in Brazil. Nutrient solution in the base of excised culms is used to allow fertilization in an environment far away from other richer in sugarcane pollen, normally in a forest away from the germplasm bank. (**a**) Biparental crosses where the panicles are inside a lantern; (**b**) Multiparental cross using a lantern and a container with nutrient solution

6 Biparental Cross

For biparental crossing, two clones or cultivars are used, one as female (pollen receptor) the other as male (pollen donator). Another way possible is the biparental reciprocal cross; both plants are pollen donator and receptor. Culms are isolated from the progenitors, and its length is adjusted by a secondary cut in order to make the female inflorescence to be a little below the male one, in other words, to use the gravity and to facilitate the pollination. The culms are immediately inserted in a container with a nutritive solution. This pair matting inside a lantern in the top, and with the culms bases inside a container with the nutritive solution is isolated from other crosses, keeping them inside a greenhouse. After the fecundation, the male culms are discarded and the females are transferred to special environments for seed maturation, for a period of about 20–25 days in Brazil. The fuzz is removed from the seeds to improve storage and germination, and kept at –50°C until the time for sowing (Cesnik and Miocque 2004).

7 Multiparental Cross

This type of cross is not used as often in sugarcane. At RIDESA, usually the base of five to eight culms from different cultivars are introduced simultaneously into a single container with nutritional solution, both previously identified as male or female. Emasculation is done in hot water without significant loss of fertility in the female plant of the cross. A lantern is inserted in the top, and all the other following

steps are identical to those described for biparental cross. The advantage of this method is the production of a large number of seeds, reducing the effect of genetic incompatibility, and to increase the recombination and general heterosis.

8 Recurrent Selection and Crossing Planning

Recurrent selection is any cyclic process of breeding that involves the production, evaluation of progenies, and recombination of its selected members. By the degree that this methodology is used in sugarcane, we can assume that is basically a recurrent selection. In this strategy, the superior clones or new cultivars will be again be crossed for generation of new progenies that will follow the same process repeatedly (de Resende and Barbosa 2005).

Intrapopulation recurrent selection (SRI) is extensively used in sugarcane in Brazil. Superior clones selected at the initial stage of family evaluation are crossed between each other (recombination, Fig. 3). Because sugarcane is a semiperennial plant, there are superpositions of generations and clones of different generations, and crossing is occurring not only between the clones of the same selective cycle. SRI strategy is a more efficient strategy in species that do not show high heterosis and/or genetic divergence. In other cases, when significant heterosis is available, reciprocal recurrent selection (SRR) must be chosen.

Recurrent selection allows a gradual increase in the favorable allele distribution by means of successive cycles of selection and recombination for the best individuals originated from the best initial progenies. This is relevant because the majority of agronomic traits are quantitative inheritance, controlled by several genes, and have low heredity and strong influence of the environment. As a consequence, the probability of one clone having all favorable alleles is very low, explaining the need of recurrent selection.

More efficient selection of superior individuals will definitely be accomplished if the selection is performed within populations with higher media or higher frequency of favorable alleles. Considering this statement, several sugarcane breeding programs have first done the selection of families before the selection of a clone (Cox et al. 2000; Bressiani 2001; Kimbeng and Cox 2003; Barbosa et al. 2004), mainly regarding traits in which heredity is based in the family mean that they were superior to the heredity of the individual plants, as happens for the culm production trait.

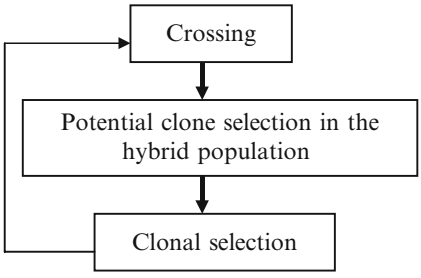

Fig. 3 Basic workflow of intrapopulation recurrent selection used in sugarcane in Brazil

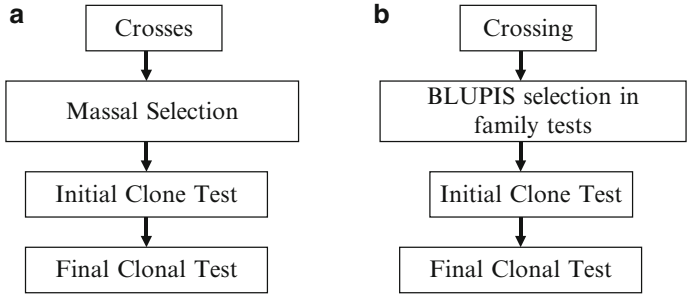

Fig. 4 Basic workflow for two different methods of selection of sugarcane elite clones. (**a**) Using massal selection; (**b**) BLUPIS selection

The scheme (a) presented in Fig. 4 has been the normal breeding procedure of RIDESA over the past years. After the crosses, massal selection in done in order to select the individuals that will be submitted to the clone tests. In other words, family evaluation data are not used for the selection of potential clones. The criteria used for the cross selection are the following: (1) Avoidance of crosses between parents; (2) Preferentially, crosses between elite clones or cultivars developed in the country or region; (3) Presence of several important agro industrial traits in the parents.

Method B presented in Fig. 3 is based on the use of a family test in experiments with replicates, but any measurement is done at an individual level. In consequence, it is not possible to predict the individual genotypic values of the potential clones by the individual BLUP procedure. However, a modified BLUP method, named BLUPIS (simulated individual BLUP) was developed by Resende and Barbosa (2006), which allows the indication of how many individuals must be selected in each family to be submitted to clone testing. The BLUPIS procedure is superior to the traditional BLUP in sugarcane and forage breeding (like elephant grass and *Panicum* spp.), where the experimental plots are harvest at once.

Quantitative genetic studies have revealed that the additive genetic variance is more important than the nonadditive variances for the majority of traits of agronomic importance in sugarcane. The main exception is for culm production, where both sources of variance seem to have similar importance (Bastos et al. 2003, Berding et al 2004).

Hogarth et al. (1981) have shown that additive genetic variance was superior for brix and number, diameter, and height of the culms. They also related the significance of nonadditive genetic variance for several characteristics, except brix and culm number. These results agree with ones obtained by Bastos et al. (2003) that also verify that additive genetic effects are as important as nonadditive effects of sugarcane agronomic traits. As a consequence, the specific combining ability (ECA) is as important as GCA.

The importance of both GCA and ECA is one important argument that supports the importance of the SRR. The SRR can be applied in two different ways: (1) inside a population, involving crosses of several individuals of one population with individuals of other reciprocals; (2) in the individual level (SRRI), involving only one individual in each population, which ones produce a good cross with high

total genotypic value and also high ECA. Such individuals originated from a superior cross are self-fecundated, producing two populations S_1 in which superior individuals will be selected to integrate one SRR program. The SRRI using S_1 is indicated for the breeding of sugarcane because the aim is to get maximal ECA beginning in the anticipated identification of better crosses by means of family evaluation experiments. The employment of autogamous individuals selected in families S_1 aim at eliminating the negative genetic load of the population and to explore a further cycle the superior hybrid combination previously identified. Because this process is cyclic, new hybrid families will be identified and explored by SRRI-S_1. This breeding strategy, using population SSR and SRRI, is further detailed by de Resende and Barbosa (2005).

Culm productivity improvement as a quantitative trait controlled by a very large set of genes, justifies the use of the classical method of recurrent selection. Through this strategy it is possible to stepwise increase the frequency of the linked alleles at each selection cycle. This traditional method is also responsible for the increase of productivity in several important crops. Presently, despite large efforts to obtain single-dose molecular markers in sugarcane, the classical breeding methods are still the more used tools to improve sugarcane productivity and quality. Urgent and thorough collaboration of interdisciplinary groups including plant physiology, functional genomics, and molecular genetics are needed to speed up this process and accomplish the actual exponential demand for renewable biofuels.

9 Heritance of Principal Characters

Skinner et al. (1987) have heritability described data for several important agronomic traits using both an individual and a family selection. Bressiani et al. (2001) performed similar studies in Brazil comparing the same genotype in different locations, and the results are compared in Table 2 below.

From Table 2 we can conclude that despite significant differences there exists a heritance between different environments. Selection using individual plants (massal selection) results in much lower heritance that family selection, with exception for brix and disease resistance. From these data we can conclude that massal selection (individual evaluation) must be used only when the trait has high heritance. Although the discrepant values exist for individual selection between Brazil and Australia, the heritance estimate based on family selection are very similar.

Other positive aspects of the selection of using family instead of individuals is that the phenotypic performance could be evaluated in different environments simultaneously since the beginning of selection phases. This aspect is of great importance if the interaction of genotype × environment is very high for the trait of interest, as exemplified by fiber content in the Brazilian sugarcane production regions. Jackson et al. (1995) suggest that the differences in the levels of calcium and zinc in the soil could possibly increase the effect of the environment in the selection. Several studies done in Brazil have shows that the effect of the environment could change the strategies of selection

Table 2 Heritance estimates based on individual plants and families (between parenthesis)

Trait						
	Australia	Hawaii	Brazil[a]	Brazil[b]	Fiji	Argentina
Cane yield (ton/ha), TCH	0.17 (0.75)		0.50 (0.78)	0.45 (0.65)	–0.48	0.1
Sugar yield (ton/ha)	0.16 (0.76)		0.53 (0.80)	0.49 (0.72)		
Culm brix	0.65 (0.90)	0.27 (0.53)	0.92 (0.81)	0.77 (0.87)	–0.43	
Tiller number	0.26 (0.90)	0.13 (0.51)	0.64 (0.83)	0.62 (0.84)	–0.53	0.06
Culm diameter		0.30 (0.71)	0.63 (0.84)	0.56 (0.84)	–0.7	0.44
Plant height	0.32 (0.84)	0.21 (0.40)	0.83 (0.85)	0.81 (0.64)	–0.54	0.24
Volume		0.10 (0.39)				
Rust resistance	0.51 (0.93)					
Smut resistance		0.56 (0.84)				

Adapted from Skinner et al. (1987) and Bressiani (2001)

[a] Same genotype evaluated at Piracicaba city, São Paulo State

[b] Same genotype evaluated at Jau city, São Paulo

(Bressiani et al. 2002; Silva et al. 2002). The last authors, for example, have observed marked changes in heritance for some traits between different locals, and concluded that better genetic yields for culm diameter and brix could be obtained in Pindorama City, whereas better gains for tillering and height were obtained in Piracicaba, two cities of São Paulo State, in Brazil, which present differences in soil and natural water availability, and differences in incidence of different biotic stresses.

It is important to test a large range of families at the original seedling stage of selection, rather than large populations of seedlings in individual experimental crosses. This is because of the importance of nonadditive genetic variance for yield of cane, which makes it impossible to predict the cane yield of seedling families. By testing a large range of families in replicated and weighted trials, it is possible to identify the families with outstanding productivity. These families are then replanted in much larger populations in subsequent years as well as undergoing selection in the first ratoon crop after weighted families in the plant crop.

Increase in family selection could be followed by individual selection, in posterior phases of breeding, comparing the individual phenotypic value with the mean of family value (McRae et al. 1993; Cox et al. 1996). Cox and Hogarth (1983) suggested better massal selection is possible if done in the ratton cane of the best families previously analyzed. A modified sequential selection method has been adopted in Australia for a long period of time (McRae et al. 1993). This selection is one form of selection between families, where the better individuals of previously chosen families are selected. The families are divided into classes, in accord with phenotipic values, selecting higher numbers of individuals chosen from superior classes, and lower, progressively in the inferior classes. This method is different from the one used at RIDESA using BLUPIS, as described by Resende and Barbosa (2006). In any case, at later stages of selection, clones are grown in larger plots and trials are often replicated. This reduces the importance of error variance, and there is a large increase in the degree of genetic determination on an individual basis.

10 Stages for the Development of a New Sugarcane Cultivar

Table 3 illustrates the possible and shorter series of events leading to a cultivar production beginning in seedling production and the following selection steps, usually done by the Federal University of Viçosa/RIDESA (www.canaufv.com.br) in Brazil.

Table 3 Phases of breeding strategy at RIDESA, Brazil, criteria evaluated at each phase is described further latter

Phases	Planting (month/year)	Evaluation (month/year)	Evaluation criteria
Crossing and seed production[a]			
Field trial 1 (FT1) Seedlings are planted in the field spaced 0.6 m between plants and 1.5 m between rows.	October/00	July/0l (cut without evaluation) May/02 (selection)	In the ratoon cane of selected families by BLUPIS[b] proceed with visual analyse: diseases, flowering, lateral shooting, culm diameter and height, number of tillers, and brix. Selection of clones for FT2. No more than 5% are selected from total population
Field trial 2 (FT2) Seedcane clone originated from families selected in FT1 are planted in rows with 6 m of length and 1.5 m between rows. Augment Federer Block is used, and 20 treatments for block with two checks in one site.	May/02	June/03 (only get data) June/04 (selection)	Selection by genotypic values (BLUPIS) for ton of brix per plot produced in plant cane and ratoon. The breeders confirm the performance of clones on the visual phenotypic analysis in the ratoon cane: morphologic characters, flowering, number of culms in the ratton, spongeous culms, and diseases. Selection of clones for FT3
Field trial 3 (FT3) Seed cane clone derived from further restriction of selected clones in FT2, are planted four rows with 5 m of length	June/04	March/05	No selection. The FT3 is used to produce seed cane (multiplication phase)
Field trial 4 (FT4) Regional assay in augment Federer Block, in 3 sites. Plots of 4 rows with 5 m of length	March/05	May/06 (only get data) June/07 (selection)	Selection by genotypic values (BLUPIS) for ton of sucrose per plot produced in plant cane and ratoon. The breeders confirm the performance of clones on the visual fenotipic analysis in the ratoon cane: morphologic characters, flowering, number of culms in the ratton, spongeous culms, and diseases

(continued)

Table 3 (continued)

Phases	Planting (month/year)	Evaluation (month/year)	Evaluation criteria
Field trial 5 (FT5) Produced seedcane. Plots of 10 rows with 15 m of length.	June/07	March/08	No selection. The FT5 is used to produced seed cane
Field trial (FT6) Produced seed cane in mills and distilleries. Plots of 10 rows with 15 m of length. 10 sites	March/08	March/09	No selection. The FT5 is used to produce seed cane
Field trial 7 (FT7) Randomize blocks, plots of 5 rows with 10 m of length in 10 sites	March/09	July/10 July/11 July/12	Technical evaluations in the first, second and third harvest. Sucrose content and cane yield. Diseases and management characters
Field trial (FT8) Maturation curve. One row with 3 m of length replicated 2 times, in 3 or more sites	March/11	April–Oct/12	Sucrose content from April to October
Beginning of farmers and industrial cultivation	End year 12		Cultivar is launched in the market

[a]April–June year 0. Germination: July–September year 0
[b]BLUPIS: Selection via simulated individual BLUP based on family genotypic effects in sugarcane (de Resende and Barbosa 2006)

The table shows a strategy used at one unit of RIDESA (UFV). The selection is done in two different selection systems: (1) precocious or median maturity, and (2) with irrigation or without irrigation. In general, this example is similar to breeding schedules at others universities in the RIDESA program. The RIDESA program yearly introduce in the field (FT1), in the year 0, about 2,000,000 seedlings derived from about 3,000 crosses. From these, only 100,000 are used for family selection. About 60,000 FT2 clones undergo evaluation. From these, about 6,000 clones were evaluated (FT4), also in ratoon crops, where only about 600 plants will be selected. In the FT6, the 600 clones will be evaluated in regional assays during 3 years, and the repeatability coefficient estimated by Ferreira et al (2005). These regional assays are realized by the seven breeder teams in seven different Brazilian States – regions). Only in Minas Gerais, the FT6 selection is followed in 34 industries that also participate with human resources for the evaluations. From these 600 clones in the national assay, initially about 20 variety candidates are planted around Brazil, but in average, only half of them will be consolidated as more profitable genotypes. The regional assays are the more confident indicators of selection efficiency. Because we are looking quantitative traits, environments are the major component of the variance, and the permanence of a superiority in one trait over different and contrasting environments allows

Fig. 5 Stages of seedling selection and production. (**a**) Seedling production: each box corresponds to a family p progeny seedlings; (**b**) Semimechanical device used to speed up seedling planting in the FT1 phase. (**c**) Strong variability shown in early phases of seedling development: rich source for breeding of sugarcane. (**d**) Individual selection to obtain clones for FT2. The *arrows* show two clones already selected for the next phase

that the selection could discard the environmental effect and indicate the real true genotype value of the trait. Each of these phases are further described below.

11 Selection in Original Seedlings on FT-1

Samples of seeds of each cross are submitted for germination testing before the whole seed set of one cross is germinated. In optimal conditions, germination usually occurs between 3–5 days, and the ideal temperature is between 25–32°C. The fertility of each female plant is also recorded, and for several genotypes is also stored in the RIDESA data bank. Panicles that are subjected to a quick drying in 48 h germinates better that other submitted to longer drying or high humidity treatments. If the results of germination testing are good, the sowing of seeds is done in wood or plastic boxes filled with special plant substrate (Fig. 5).

12 FT2

Five percent of FT1 selected clones undergo their first clonal multiplication. Subsequently and in the FT2, two reference varieties will be used as a control, planted randomly in each block, which are constituted by the varieties more cultivated in the region. In some universities affiliated with the RIDESA group, Federer Block are used with 20 treatments per block, where the other clones are planted without experimental design due to the large number of clones under evaluation at this phase (in RIDESA, annually 600,000). In the last case, only the visual selection is done. The FT2 evaluation will be repeated with the ratoon crop, and the average of the evaluations of the two observations will be used to compute genotypic value. The genotypic values estimated by BLUP are done based on brix ton/ha (BTH). For calculation of BTH, three independent brix determinations (hand refractometer),

total tiller number, and culm weight (ten culms) are required in each plot. Later, the breeders return to the field with all data, and added a last visual inspection, and select the clones, taking into account negative morphologic characters and disease resistance in 10% of the superior clones.

13 FT3

This phase multiplication is done to establish the FT4 in at least three different places, with sharp differences in climate and soil. The selected clones are planted in June (dry period), with irrigation, and after 9 months of growth, enough cane seed is produced to perform the FT3 phase.

14 FT4

Selected clones at FT2 and propagated on FT3 (about 10%; 6,000 plants) will be planted in bigger plots (4 rows with 5 m of length), in an augment Federer Block. Each block will now have two controls as in FT2, and statistical analysis will be applied. Several technical evaluations in the first harvest and second harvest (ratoon cane) are done: sucrose content, cane yield, number of culms and average culm weight (from 10 culms). The use of three sites to evaluate reduces the environment effect in the phenotype and in the experimental variance, increasing the degree of genetic determination on an individual basis.

15 FT5 and FT6

The clones selected at FT4 will be multiplied in order to produce cane seed to install experiments at least in 10 different contrasting places.

16 FT7

The selected clones on FT4 are introduced into various mills and distilleries for each breeder team of RIDESA, to evaluate the clones under natural agronomic management systems and different environments. Experiments are collected for three successive harvests. This operation may be done after prior straw burning and weighting of plots using a sugarcane loader with a load cell or dynamometer coupled to its grab (Fig. 6).

Fig. 6 Quick measurement of total culm weight of a plot in FT7 using a wireless controlled dynamometer

17 FT8

Maturation curves are performed regionally in several places. Other morphological features and agronomic performance in the field are also recorded. During all phases, the clone's resistance to the most relevant diseases is assessed in the field.

18 Active Clone Exchange Between Breeder Teams

One important feature that contributes to the success of the RIDESA breeder program is the unrestricted reciprocal exchange of clones between the seven breeder teams spread across seven federal universities members of the RIDESA consortium. Every year, each member sends all other members about 50 new clones that have been previously developed and selected in their own states. Such logistics strengthen sugarcane breeding and increase the offer of new clones for mills and distilleries that have cooperation agreements with RIDESA. This also involved the testing of varieties in extremely contrasting regions between 9°S and 25°C, involving presence or absence of cold and water stress, different climates, soil conditions and agronomic management systems. This wide testing network allows more successfully evaluation of genotypic value of traits under focus.

19 Cultivar Launching Phase

In fact is still a multiplication field where, in RIDESA, means about 600 clones yearly are evaluated nationally. Around 20 cultivars candidates will be selected for the marker and spread for testing in a larger group of industries, a last selection will perhaps retain half of these initial cultivars, which slowly will be adopted in the Brazilian market. Actually any mills or destilaries cultivate, in

average, at least ten cultivars, in order to protect itself from epidemics and also to have a long harvesting period.

20 Breeding for Physiological Traits that Affect Biomass Production

Few reports are available about this topic, but some recent efforts are changing this trend (Berding et al. 2005; Rae et al. 2005; McCormick et al. 2008). Leaf area and inclination, net rate of photosynthesis, duration of growth period, are each governed by multiple genes and important factors that could act together to improve yield and quality (Bull and Glasziou 1975). He found that high photosynthesis are higher in recently expanded leaves of young plants when temperatures are high (summer). In contrast, mature leaves or young leaves in more cold temperatures have assimilation rates similar to C3 plants. This was attributed to lower stomata conductance, characteristic of sugarcane that contributes to optimize its water use efficiency. He proposed that increase in temperature tolerance could contribute to selective advantage, or even higher photosynthesis, under lower temperatures. That author also observed that *S. officinarum* and commercial cultivars had higher dry matter production than certain *S. robustum* and *S. spontaneum* cultivars, although photosynthetic differences between varieties were similar to those found within a single variety. Because that leaf area showed a high correlation with dry matter yield during the first 5 months, the author proposed that rate of leaf area production in the beginning of plant growth is of greater importance than differences in photosynthesis rate. These parameters have not been used in breeding, in part because several of them are of low inheritance.

Heavy tillering has been considered a desired feature, based on the argument that could result in increased leaf area and tiller survival. This parameter has been used often in breeding selection, since its easiness to measure. Rocha (1984) has compared the tillering of three cultivars (CB41-76, NA56-79 and IAC-205) during the cultivation in two different periods in its respective ratoon crops. The maximum number of tillers was observed 6 months after the sprouting (October for the first crop, and January for ratoon plants), both in cana or ratoon crop. The tillering occurs until the appearance of tertiary tillers, and rarely, quaternary tillers. Since the sprouting until the harvest (September–October in this study), plant and ratoon harvest have almost all tertiary tillers dead, and in some cases some primary also, having the secondary tillers the high rate of survival. Prado (1988), analyzing the cultivars SP70-1143, IAC52-150 and NA56-79, evaluating the tiller number and culms in the first and second harvest, has observed occurred reduction of 50%, 30%, and 40% for the cultivars cited, respectively, at the end of productive cycle. These results suggest that sink competition between culms could be an important factor driving productivity, number of harvest and sugar production and care in breeding for overvalorization of sprouting could have negative effects in productivity.

Stevenson (1965) has exemplified well the quantitative inheritance of Brix. Whereas in progeny, the Brix values have ranged from 25% to 27%, in their parents these values do not exceed 21–22%. There is no consistent evidence that increase in sink strength, like sucrose concentration in sugarcane, could increase photosynthesis in leaves of sugarcane. Photosynthesis in C4 plants is equally or even less sensitive to water stress than its C3 counterpart in spite of the greater capacity and water use efficiency of the C(4) photosynthetic pathway (Ghannoum 2009). It is well observed in Brazil that drought and low temperatures near the maturation stage could trigger sucrose accumulation in stalks (Casagrande and Vasconcelos 2008). One alternative interpretation, still not tested experimentally, could explain that positive effects of abiotic factors are responsible for the decrease in sink competition: decrease in water availability could quickly decrease leaf expansion, internodes, root and tiller growth, and triggering senescence, (Guo and Gan 2005), which results in mobilization and transport of organic molecules from senescent organs to culm, allowing that more sucrose could be stored in the stalks. However, sugar and fiber content are quantitative traits and are controlled by a variable interaction genotype environment and multiple genes, and no so simple hypothesis could explain this complex process, being necessary to characterize the chain of physiological factors involved, to help the breeders to take a step further in the sugar content improvement (Singels et al. 2005).

Lodging in sugarcane is considered to exist when the culms have a vertical angle higher than 60–70° (Amaya et al. 2000). Several factors could contribute to increasing lodging, such as height and diameter of stalks, growth profile, environmental factors such as winds, nutritional deficiencies, or elevated culm weight (Singh et al. 2000). In Australia, lodging was associated with “slow down” in biomass accumulation in mature sugarcane crops, which significantly decreased both fresh cane yield (11–12%) and commercial cane sugar (CCS) content (Singh et al. 2002). Sugarcane is known to absorb more Si than any other mineral nutrient, accumulating approximately 380 kg/ha of Si, in a 12-month old crop, a fact that reduced lodging, improved leaf and stalk erectness, and also tolerance to biotic and abiotic stresses (Savant et al. 1999). Decrease in Si with stalk growth was also reported, and could perhaps explain perhaps why high culm height could improve lodging (Kaufman et al. 1981).

Vermerris et al. (2002), analyzing maize lignin mutants, have suggested that plants with reduced lignin content could have high susceptibility to lodging, but this was not tested. It is reasonable to believe that there are limits of genetic manipulation of culm lignin content to produce better biomass for biofuels. High nitrogen fertilization is associated with plants more susceptible to lodging. Balanced fertility helps to decrease lodging. Straw from potassium deficient plants appears to be more brittle (hence greater tendency to lodging) than those fully supplied with potassium. Plant morphological (structural) characteristics such as plant height, wall thickness, and cell wall lignifications can affect the ability of the plant to resist a lateral force. Tall plants have a higher tendency to lodge than short plants. A small change in plant height can have a strong influence on lodging. Studies about expression of lignin biosynthesis enzymes show that high correlation between gene expression of these genes to stem rigidity and lodging character in wheat occur (Ma et al. 2002).

Comparative anatomy under course in our group have show that the patters of vascular bundles structure and sclerenchyma tissue distribution and thickness of secondary cell wall could be different between different genotypes, and could reflect changes in lodging. How silica and nitrogen fertilization could affect this structural traits of the culm are not known.

21 Limitations of Cellulosic Ethanol Production from Sugarcane Bagasse

The sugars in well washed sugarcane bagasse are mainly polysaccharides, such as cellulose and hemicelluloses, which are not readily available for ethanol production, being anecessary pretreatment before saccharification and fermentation. Pretreatment is a process during which the sugarcane bagasse is subjected to chemical and/or physical agents with the aim of improving the rate and the extent of cellulose hydrolysis. This is achieved by an overall loosening of the cell wall structure, reduction in lignin content deconstruction of hemicelluloses, and reduction in the crystalloid structure of the cellulose (that reduce binding of cellulase to cellulose), while minimizing the formation of degradation products that could interfere with the microorganisms used during fermentation, such as furfural, ferulic acids, and coumaric acids, among others (Sun and Cheng 2002).

In particular, lignin appears to have a strong negative effect on the ability of cellulolytic enzyme complexes to digest plant biomass to fermentable sugars (Chang and Holtzapple 2000). Draude et al. (2001) showed that a removal of 67% of the lignin from soft wood pulp resulted in a nearly threefold increase in the yield of reducing sugars, and a twofold increase in the initial hydrolysis rate. Lignin appears to have two primary effects on the enzymatic hydrolysis of cellulose within this matrix: it prohibits cellulose fibber swelling, which reduces surface area access to the enzyme (Mooney et al. 1998), and cellulases irreversibly adsorb to lignin, thus preventing their action on the cellulose (Chernoglazov et al. 1988; Converse 1993; Palonen et al. 2004). This effect results in more enzymes being needed, contributing for the noncompetitiveness of this technology at the moment. During pretreatment, hemicellulose sugars may be degraded to weak acids, furan derivates, and phenolics such as ferulic acid and others that are linked to arabinose and galactose. These compounds inhibit the later fermentation, leading to reduced ethanol yields. The production of these inhibitors increases when hydrolysis takes place at higher temperatures and higher acid concentrations. Different chemical pretreatment could be used but there is no consensus about which one is the best process for bagasse pretreatment. We also do not know anything about the effect of genetic variability in cell wall composition in the bagasse in the relative efficiencies of these several chemical method options (Sendelius 2005; Prior and Day 2008).

Hydrolysis of stover from *bm* maize mutants, which have reduction and changes in lignin composition results in an increase of 40–50% sacharification process

efficiency, compared with control plants. Similar results with sorghum *bm* mutants suggest that biomass conversion efficiencies can improve significantly by making specific modifications to the lignin content and lignin subunit composition (Vermerris et al. 2007).

22 Breeding Sugarcane for Better Efficiency of Cellulosic Ethanol Production

The basic composition of a mature sugarcane culm is presented in Fig. 7.

Biomass quality is heavily influenced by cell wall composition and structure (determined by content and composition of lignin, cellulose, hemicellulose, and the way they are cross linked), whereas biomass yield is determined by agronomic traits such as plant height, stalk diameter, number of leaves, disease and pest resistance, and lodging susceptibility, and can be manipulated through crop management practices. We have already briefly discussed biomass yield previously. Quality actually seems to be a major concern, because the high cost of enzymes used in sacharification, and the degree of how changes in cell wall composition affect the amount of this enzyme needed for enzymatic hydrolysis (Anderson and Akin 2008).

Dry weight sugarcane bagasse is similar, in general terms, to the composition of stalks of other plants, basically composed from 50% α-cellulose, 25–30% hemicelluloses, and 10–25% delignin. However, sugarcanes, as grasses, have different cell walls than Dicots. Non major differences for cellulose content in primary and secondary walls between Dicots and Monocots were observed but dramatic differences in hemicelulose composition are observed. In primary cell walls of grasses, we can see 4–8 times more xylans, high levels of MLG (mixed linked glucans), absent in Dicots, and very low levels of xyloglucan, mannans, and glucomannans and pectin. However, much higher levels of phenolics, and often a double content

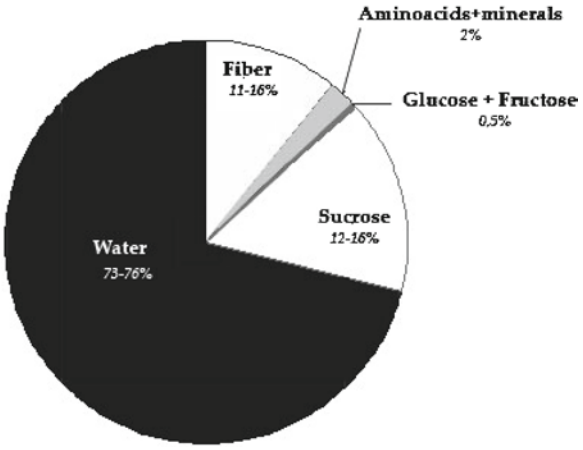

Fig. 7 Major components levels in mature sugarcane culms. Values are relative to fresh weight basis

of lignin in secondary walls occurs. Principal monosaccharide released from the cell walls of grasses are glucose, xylose, and arabinose. Arabinose levels decline markedly during plant maturity, leaving glucose and xylose as the major sugars. Lignin structure is also different in grasses, having more H residues (more coumaryl derivatives) (Vogel 2008). However, the exact values for of the different cell wall fractions of sugarcane are still unknown.

While production of cellulosic ethanol from pure biomass, in general terms, is feasible from an energy-balance perspective, its production is generally considered as not economically competitive at the present time. Along with improvements in bioprocessing, enhancing the yield and composition of the biomass has the potential to make ethanol production considerably more cost effective (Wyman 2007). However, this seems to be different for sugarcane because the simultaneous and cheaper use of sucrose in parallel, as residual sucrose in the bagasse, make the biomass conversion more feasible. Dedini, the major industry building sugarcane distilleries in South America, began the building of a big plant to produce ethanol from sugarcane bagasse in 2002, initially at the cost of US$0.40/L ethanol. In the following years with changes, completed, the cost could fell to US$ 0.27/L, a cost that is still double that of alcohol production directly from sucrose. Due to oscillations in exchange rates, the economical viability at this stage exists when the cost US$ 0,42 or less. Intensive research is in progress to decrease the cost of this process, and soon will be changed. The new plant is able to produce 43 million of litters ethanol per year. Dedine was recently bought by Abengoa, a giant Spanish company for the generation of energy, which also has several plants working in the USA (Biocom 2007). This seems to be the first technology specific developed for cellulosic ethanol production from sugarcane bagasse.

The traditional process of breeding selection of sugarcane has always been focused on sucrose production. With the actual demand of generation of energy at the mill using bagasse and crop trash (leaves and tops), the cultivar selection process should take into account the trash and fiber that can be produced by the different clones. Two potential ways to improve generation of energy from sugarcane are, first, to maximize trash recovery from the fields to enhance biomass, and second, to produce new cane varieties with higher biomass as a potential source of raw material (Paes and Oliveira 2005).

The traditional selection strategy evaluate the clones in regard to cane yield (ton/ha), CCS (%FW), and sugar yield (ton/ha). The calculation of CCS which provides an estimate of the percentage of recoverable sucrose from cane takes into account the results of brix (total solids in juice using a refractometer) and from sucrose in juice (using a polarimeter), as well as fiber percentage determination. This calculation is usually done to estipulate the farmer payment by the quality of sugarcane, but it is also used in breeding. In the field, the brix measurement is done for the first selection phases. About five culms, randomly selected, at least in three points have the juice extracted with a Hawaiian borer, and always mixed together in the same correspondent internode. Two or three drops of this mixture are then used in the refractometer (Cesnik and Miocque 2004). Because the tillering temporal processes are variable between species, and because the majority of the tillers die, some effect

of tillering time profiling in mean brix values seems quite obvious, but, in fact, is not measured or rightly estimated during selection. Sugarcane has a variable propensity to produce suckers, late-formed tillers in mature crops. These suckers are low in sugar content and result in a dilution of the sucrose content of the harvested crop, and not taken seriously in the breeding calculations (Berding et al. 2005). These deficiencies in the methodology used could explain a positive selection for sugar content in detriment of cane yield, and could also suggest that actual methodology can cause errors in the calculation of total sugar production by area.

Major breeding efforts did not take in the consideration of cane yield, the tops of the plants that remains as mulch trash. The amount of trash residue from sugarcane harvesting depends on many factors, for instance the harvesting system, topping height, cane variety, age of crop, climate, and soil among others. Its value is around 14% of the stalk mass (Paes and Oliveira 2005). Because sugarcane is one of the more productive crops, it also produces more large content in trash biomass for cellulose ethanol production, and enormous potential of generation of cellulosic ethanol.

No new concepts, but new breeding efforts in the direction of energy cane are presently becoming more conspicuous in sugarcane community improvement. It seems that a general skepticism exists based on the assumption of a mandatory and stark negative correlation between the sucrose and fiber content. This new strategies of breeding have been originated in good part from the work of two Caribbean breeders, from Mauritius (Autrey and Chang 2007; Mauritius Sugar Industry Research Institute) and from Barbados (Rao 2007; West Indies Central Sugarcane Breeding Station), who were pioneers in the defense of the need to change breeding in order to get more energy from sugarcane biomass. Based on their arguments, we can divide sugarcane breeding into four major groups, as is present in Fig. 8.

The concept of energy cane has as underlying statement that an efficient energy conversion system is available, and takes into account only theoretical calculations

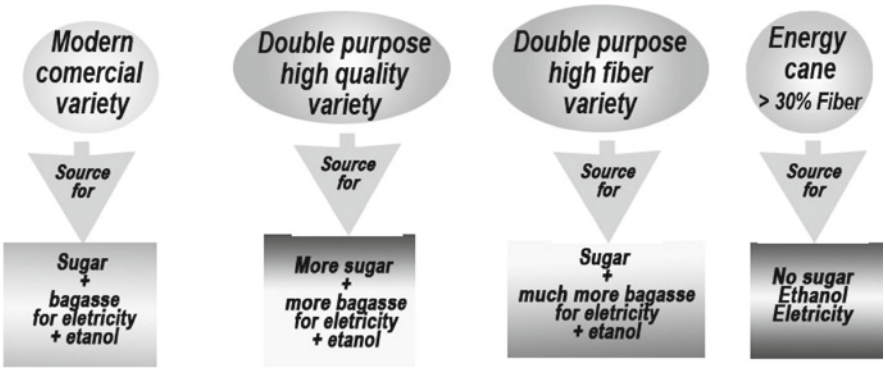

Fig. 8 New breeding objectives of sugarcane breeding for lignocellulosic ethanol. (Modified from Autrey and Chang 2007)

(Rao 2007). More conceivable at this moment is to consider the possibility of breeding sugarcane for a double purpose, enzymatic hydrolysis and pyrolysis. Both seem achievable options at short and medium term respectively.

In Mauritius, Autrey and Chang (2007) were successful producing a multipurpose sugarcane, as show in Table 4.

Similar results were obtained by (Rao 2007) in Barbados, as show in Table 5.

These tables illustrate that it is possible to obtain multipurpose sugarcanes (WI79460 and WI79460). These varieties, despite a lower culm brix and sucrose, have more cane yield, which allows that the potential total sugar/ha does not decrease significantly at the same time as it produces almost three times more fiber. The success of both efforts came from the crosses of elite cultivars with *S. spontaneum*, probably due increases in the frequency of genes responsible for higher tillering and biomass production, and a heterosis effect. Bressiani et al. (2005) related that CTC breeding in Brazil has also been successful to select varieties for high biomass amount simultaneously the high sucrose content but no or anticipated commercial varieties are yet available.

Table 4 Mauritius multipurpose sugarcane cultivars

Varieties	B77602	WI87718	WI79460	WI79460
Cane ton/ha	77.6	59.7	112.2	125.4
Juice brix	22.9	22.6	19.4	16.1
Purity	89.1	89.5	65.8	73
Brix % cane	19.4	18.5	14.2	12.2
Pol % cane	17.3	16.6	9.3	8.9
Fibre % cane	14.8	18	26.9	23.9
Brix ton/ha	15.1	11.0	15.9	15.3
Fibre ton/ha	11.5	10.8	30.2	30.0
Dry matter (brix + fiber) ton/ha	26.6	21.8	46.1	45.3
Tops %	17.8		19.3	17.3
Biomass (C + T) ton/ha	91.4		133.9	147.1

Table 5 Barbados multipurpose sugarcane cultivars compared with a common modern cultivar (M52/78) (Rao 2007)

Variety	Pol % cane	Fiber % cane
WI 99901	17.3	14.0
WI 99940	17.9	18.4
WI 96912	19.9	17.5
WI 96911	20.3	13.1
WI 99906	21.2	14.0
WI 96904	20.5	13.0
WI 99902	20.9	12.7
WI 99920	22.3	12.1
M 52/78	14.3	10.7

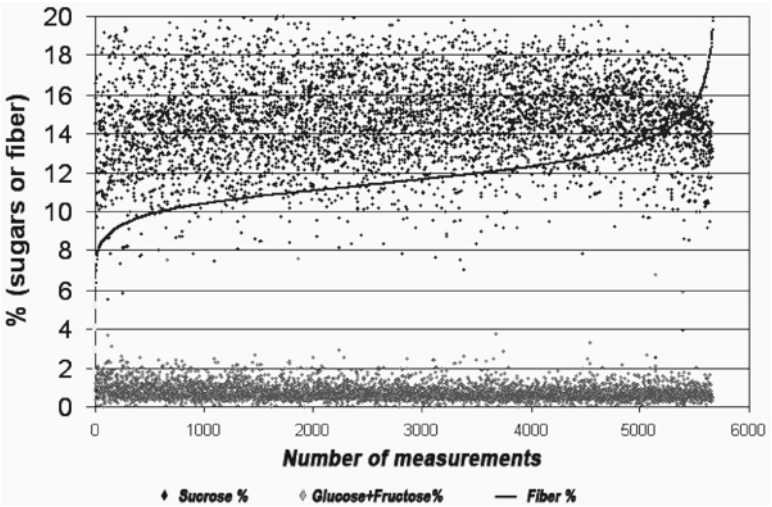

Fig. 9 Data showing percentage of sucrose and glucose + fructose in relation to fiber content in 241 genotypes in three different harvests occurred in 42 different miller sugarcane fields. These data are distributed in relation to fiber content (*black line*)

Our data regarding three successive harvesting 241 genotypes, with information about fiber and levels of sucrose, glucose + fructose, cane and sugar yield, allow us to confirm the absence of negative correlation between sucrose and fiber content, as shown in Fig. 9.

The large data set produced in our experiments show that correlations (r^2) between sucrose in and glucose–fructose (0.74), and fiber % (−0,14), and to cane yield (0.06). These results suggest us that among certain limits, it is possible to increase fiber content without penalizing sugar production.

Because lignin content could have a negative effect on ethanol production, we began to characterize lignin content variation in order to analyze the possibility to use breeding to manipulate lignin amount (Fig. 10). Figure 10 shows initial characterization done in one population of elite clones that were in the selection process.

This initial experiment shows us that there could be found large variation in lignin content (from 5 to 18%) in sugarcane bagasse, and indicate that there is enough variability between the clones and cultivars for production of celulosic ethanol. Our group is actually using this contrasting material and comparing the yields of saccharification and fermentation. However we are still in the preliminary stages and need to further analyze the genetic control of fiber composition. Several new crosses between our elite clones and *S. spontaneum* and *S. officinarum* are under evaluation in order to measure variation lignin content and composition. However, further careful analysis will be needed in order to show the limits of change in lignin content changes that could be achieved without significant impact in biotic stress resistance, and also, to test saccharification efficiency between contrasting clones.

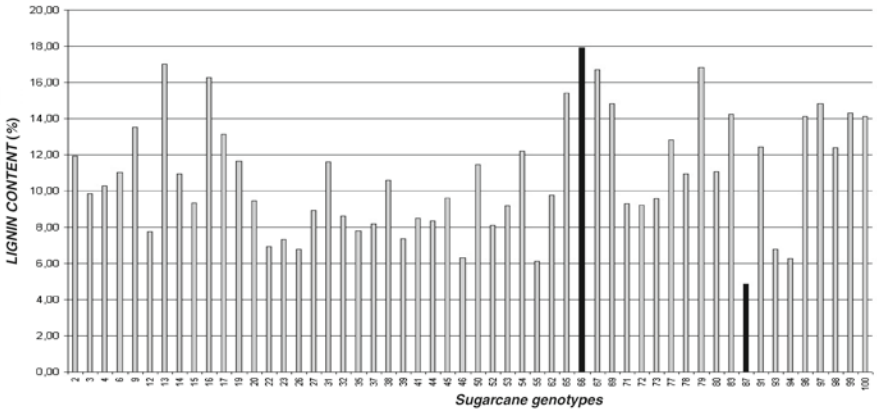

Fig. 10 Variation in lignin content in cell wall extractive in one set of clones under selection at RIDESA-UFV, at maturation. The two black bocks are the extreme values observed in the population analyzed (Barbosa et al. unpublished results)

24 Development of High-throughput Methods for Characterization of Cell Wall Traits Important for Cellulosic Ethanol Production

Classical methodologies of lignin quantification by the Klasson method and composition using HPLC are not feasible to be used with large sample sets because long time frames and high costs are prohibitive (Silverio et al. 2008).

Other analytical alternatives must be validated for speed up this process. One very promising technique which is very sensible to discover silent phenotypes in plant cell wall mutants, is Fourier transformed infrared spectroscopy (Chen et al. 1998). However the results have high standart desviation, and sensibility is low. Additionally, analytical pyrolysis, already used to analyze lignin composition in wood (Barbosa et al. 2008; Silverio et al. 2008), allows analysis of minute amounts of lignin (around 100 μg), allowing unequivocal molignol fragment ions identification and relative quantification for several components. Besides high sensibility, this technique is quick and feasible without lignin purification (Galletti and Bocchini 1995). We have established a quick Py-GC-MS protocol in just 20 min for sugarcane, allowing confident identification and relative quantification of about 16 lignin components (Lopes et al. 2010). Further improvements are underway, now using a GC-TOF, that furthermore, to decrease time, needs to less than 10 min per sample, allows better deconvolution (500 scans per second), and would permit an increase in the number of phenolic compounds identified and quantified in the lignin or in other components of the cell wall screening tool. Because APCI-Q-TOF is also available in our laboratory that could be linked to our GC, future attempts will be to better describe lignin structure and not solely lignin composition.

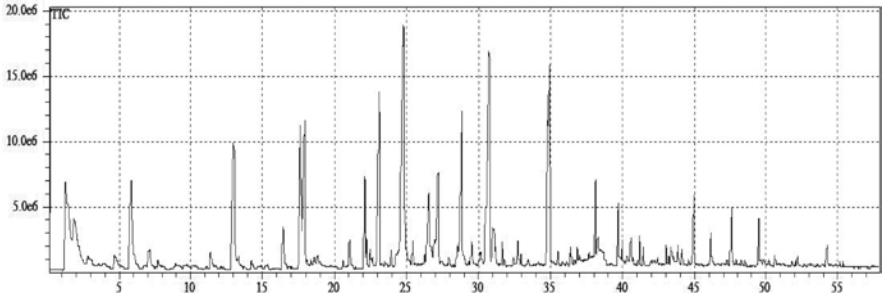

Fig. 11 Total ion chromatogram of Klason lignin Py-GC-MS analysis from the variety RB86-7515 at 450°C

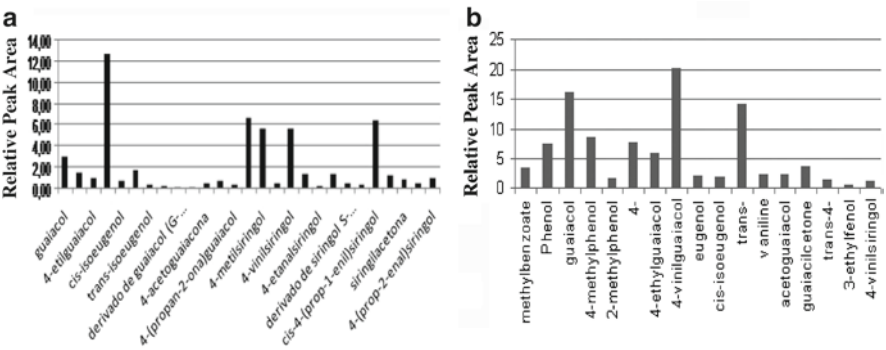

Fig. 12 Lignin composition determined by Py-GCMS. (**a**) Pyrogram from lignin extracted by Klason method from variety RB86-7515 a 450°C. (**b**) Pyrogram from pure homogenized root dry power from Arabidopsis. This quantification was validated by nitrobenzene oxidation method (NBO)

Figure 11 shows a typical total ion chromatogram of a lignin analysis of sugarcane bagasse done with pyrolysis-GC-MS.

Good separation of several peaks could be seen, and deconvolution and spectral analysis of fragments of each peak allow the unequivocal identification and relative quantification of 16 lignin components (Fig. 12).

As mentioned before, the S/G ratio has profound effects in ethanol production efficiency of biomass. Table 6 gives us some idea of its variation within clones without significant changes in lignin.

The results above have shown that no significant correlation can be seen between S/G rations and lignin content and no wide variations were found for these plants. However, studies with *Populus* show that small contrast in plants for composition and lignin levels results in sharp differences in the acid hydrolysis (Davison et al. 2006), despite the narrow variation, between 22.7–25,8% for lignin content and S/G ratio among 1.8–2.3%. Our results have shown that our clones studied show only 8% relative difference for lignin content, and for S/G ratio, 21.74%. This similar

Table 6 Comparison between lignin content and S/G ratio in clones analyzed for lignin content in 200, in one population of 288 evaluated clones. S/G ratios were measured only in ten clones (Barbosa et al. unpublished results)

	Klason lignin(%)	S/G ratio
Class I lignin content		
146	22,6371	1.38
133	22,6494	1.23
58	22,6774	1.3
166	22,6782	1.32
53	22,7169	1.25
Class II lignin content		
87	23,8285	1.08
8	24,0813	1.31
349	24,1626	1.36
321	24,4831	1.38
50	24,7027	1.22

variation (lower for lignin content) suggests that could also result in significant differences in the acid hydrolysis as determined by Davison et al. (2006). Risk of negative effects of changes in lignin composition is very low, since several studies addressing diversity of natural lignin chemistry have revealed that the flexibility of the lignin polymerization process allows plants to tolerate substantial changes in their lignin composition and/or incorporate phenolics other than the three common monolignols into their lignin (Ralph et al. 2004).

We have made an extensive comparison about the determination of lignin composition using dry organ powder, cell wall extractive, or lignin extracted by the Klasson method in sugarcane. Additionally, we can further speed up the determination of S/G, using a ball milling machine for 96 deep wells using very small amounts of tissue, and preparing a simplified cell wall extraction. This approach could achieve the yield of 300 samples a week. We believe that our new attempts with GC-Q-TOF, a larger number of unidentified phenilpropanoid metabolites will be added to this group of identified compounds, and the use of quick PCA analysis of the ion chromatograms will speed up the discrimination for genotypic differences in some cell wall constituents. Coupling of strategies such as using FTIR as a first discrimination screening and neural network analysis (McCann et al. 2007), as we are engaged with Prof. Buckeridge (USP, São Paulo), could bring us unprecedented power to cell wall phenotype discovery, and to better help breeders achieve greater biomass for biofuels in sugarcane. Silent cell wall phenotypes could then be discovered and further characterized, extending the capabilities to understand the structure, synthesis and disassembly of the cell wall of sugarcane and other feedstock grasses important for biomass energy. High throughput and new methods are considered key points in the scientific development needed to turn cellulosic ethanol an attractive economical investment (DOE 2006). At this moment it is very important that functional genomics efforts could be extended in sugarcane. In this regard Brachypodium distachyon (L.) Beauv it is a useful model grass plant, since its small genome (~300Mbp), complete genome sequence, a small physical stature, self-fertility, a diploid genome, a short life cycle, simple growth requirements and relative easiness of transformation with Agrobacterium tumefasciens. More near genetically to

sugarcane that rice and maize, its cell wall characteristics as monocot make this plant as an ideal system to study cell wall genes that affects sugarcane bagasse recalcitrance.

25 Tools Needed to Speed up Sugarcane Breeding

Use of DNA RFLP analysis on basic and commercial germplasm allowed the separation of the three basic species, *S. spontaneum*, *S. robustum*, and *S. officinarum* (Grivet et al. 1996). Specific characterization of the strong molecular differentiation between *S. officinarum* and *Erianthus arundinaceus* was undertaken using RFLP markers (D'Hont et al. 1996). Several other types of molecular markers have similar AFLP and SSR are in use mainly for characterization of genetic diversity, genome characterization, construction of genetic maps, and QTL mapping (Da Silva et al. 1995; Jannoo et al. 1999; Lima et al. 2002; Selvi et al. 2005; Raboin et al. 2006; Oliveira et al. 2007). However, speed and cost of analysis limit the application of current marker technologies for sugarcane molecular breeding, diversity, and identity testing. TRAP markers for assessing genetic diversity and interrelationships in sugarcane germplasm collections were recently used. Thirty genotypes from the genera *Saccharum, Miscanthus*, and *Erianthus* were used, and results using primers for only six genes, followed by application of multivariate analysis, allowed the complete separation of genotypes (Alwala et al. 2006). Other more modern technology, Diversity Arrays Technology (DArT) combines the ability to identify various types of DNA polymorphism with the low cost and high throughput of the DNA microarray platform. Once established, DArT offers a tenfold gain over other technologies in terms of marker throughput and assay cost (Heller-Uszynska et al. 2007).

There is common interest from both the academic and private sectors to participate in a collaborative effort to establishing a draft reference sequence of the sugarcane genome, that at the present time (Sugarcane Genome Initiative, http://sugarcanegenome.org) include researchers from Australia, Brazil, the United States, France and South Africa. The complete sequence of sugarcane genome seems to be unfeasible, with the actual sequence technology, since sugarcane is a complex polyploid with a big genome size of 10,000 Mb (D'Hont 2005). The efforts are just beginning to construct this research network, focusing in cloned BACs enriched with interessing genes.

Studies of the genomic syntheny in sugarcane in the genomic region of Adh1 gene, were already conducted in sorghum, rice, and mayze. Sequence of this region in *S. officinarum* and *S. spontaneum* haplotypes showed perfect colinearity and gene structure conservation. High colinearity was also found with sorghum and rice. Sorghum bicolor is considered the closest sugarcane relative studied to date and shows a high macrosynteny with sugarcane (Dufour et al. 1997; Asnaghi et al. 2000). Since now is disponible the complete full genome sequence of sorghum, unprecedented tools for genomic and functional genomics become available that will help us to construct high density genetic maps that associated with a high

number of future molecular markers will soon make marker-assisted sugarcane breeding a reality, a fact that today is still incipient.

26 Coproducts from Cellulosic Ethanol Production

Biotechnology used to understand biosynthesis and deconstruction of biomass will enhance ethanol yields and will open the door for generation of new products or cheaper industrial raw materials, and agronomic and industrial technologies for better recovery of these materials and sucrose. Ethanol, organic acids (lactic acid, citric acid), amino acids (lysine, glutamate, phenylalanine), sugar alcohols (sorbitol), and many fine chemicals (enzymes, penicillin) are examples of products derived from renewable resources (US-DOE 2006).

The aromatic compounds might be produced from lignin, whereas the low molecular mass aliphatic compounds can be derived from ethanol produced by fermentation of sugar generated from the cellulose and hemicellulose degradation. Vanillin and gallic acid are the two most frequently discussed monomeric potential products which have attracted interest (Walton et al. 2003). Vanillin is used for various purposes including being an intermediate in the chemical and pharmaceutical industries for the production of herbicides, antifoaming agents, or drugs such as papaverine, L-dopa and the antimicrobial agent, trimethoprim. It is also used in household products such as airfresheners and door polishes. Hemicelluloses are of particular industrial interest because these are a readily available bulk source of xylose from which xylitol and furfural can be derived. Xylitol is used in place of sucrose in food as a sweetener, has odontological applications such as teeth hardening, remineralization, and as an antimicrobial agent, additionally it is used in chewing gum and toothpaste formulations (Roberto et al. 1999). Various bioconversion methods, therefore, have been explored for the production of xylitol from hemicellulose using microorganisms or their enzymes (Nigam and Singh, 1995). Furfural is used in the manufacture of furfural phenol plastics, varnishes, and pesticides (Montane et al. 2002). Depending of the development of biorefineries, sugarcane breeding must also attend this demand.

27 Transgenic Plants and Mutants and its Potential to Contribute to Better Effiency of Cellulosic Ethanol Production

Sugarcane transgenic was initially established by Bower and Birch (Bower and Birch 1992). Zhang and Birch (1995) got transgenic resistance against scald disease, as well as resistance to mosaic virus strain E (Gilbert et al. 2005). Others groups had transgenic sugarcane resistant to herbicide (Falco et al. 2000; Leibbrandt and Snyman 2003). Several groups produced transgenic plants resistant to the stalk borer *Diatraea saccharalis* (Arencibia et al. 1997; Braga et al. 2003; Falco and

Silva-Filho 2003). Transgenic variety RB855156 transformed with proline biosynthetic gene were more drought tolerant (Molinari et al. 2007).

Transgenic plants' changing cell walls have been shown to affect digestibility and ethanol production. Transgenic festuca expressing ferulic acid esterase (FAEA) targeted to the vacuole, showed increased digestibility and reduced levels of cell wall esterified phenolics (Buanafina et al. 2008). Evidence comes from the *brown midrib, a maize, and sorghum* mutants in which cell wall composition is altered. These mutants are available in both maize and sorghum and are easily recognized by the reddish-brown coloration of the vascular tissue in the leaf blade and sheath. The *Bm3* gene was shown to encode the enzyme caffeic acid *O*-methyltransferase (COMT). Several of these mutations affect lignin biosynthesis: maize bm1 mutation has been shown to affect the lignin biosynthetic enzyme cinnamyl alcohol dehydrogenase (CAD). *Brown midrib* (*bmr*) mutants of sorghum (28 mutants) show altered cell wall composition, particularly relative to lignin subunit composition, and some have superior forage quality (Porter et al. 1978), with some occurring in COMT gene. Hydrolysis of its stove results in higher efficiency of saccharification. Lignin can be engineered with large changes in H/G/S ratios. Frequently other undesirable phenotypes could follow as, dwarfing, collapse of vessel elements, and increased susceptibility to fungal pathogens, etc. The Dixon group (2007) produced transgenic alfalfa by independently down regulating 6 lignin genes, achieving the doubling in saccharification efficiency (Chen and Dixon 2007). Expression of an isomaltulose target to vacuole of culm sugarcane, produces sugarcane with the double sugar content, due to high-level isomaltulose accumulation with an unexpected absence of decrease in sucrose content. This phenotype is accompanied by delayed leaf senescence, increased photosynthesis (Wu and Birch 2007). This study demystifies the interpretation that there are osmotic limits so narrow to allow further increases in sucrose accumulation in sugarcane.

28 Emerging New Market for Energy and Sugarcane Bagasse Use

Lignocellulosic biomass can be converted to ethanol by hydrolysis and subsequent fermentation. Thermochemical processes can also be used to produce ethanol: vapor or gasification followed by generation of electrical power, or by pyrolysis (Kumar et al. 2008).

In 2006, Brazil produced more than 406 million metric tons of sugarcane, and a further 15% could be added as crop trash that is not yet used for energy production (Paes and Oliveira 2005). This production is about a quarter of the world's production, with more than 50% of the sucrose being used in the production of ethanol. The sugarcane bagasse can provide all energy required for processing the sugarcane and several mills are already selling generated extra energy. Actually, 343 millers in Brazil produce about 3,079 MW/h, from bagasse, but currently only 508 MW/h are sold and distributed outside the country, which is done

by only 48 millers (Nascimento 2008). One demand also has increased in other industries to use bagasse to generate vapor or gas, for example the citrus industry among others, which buy bagasse for their own energy use and is not computated in the cited estimations. In Brazil, bagasse is a commodity (Hassuani et al. 2005). Despite the extensive use of bagasse to produce eletricity, the amount used corresponds only to 20.7% of annual bagasse produced at the Center-South region, and 31% of the Northeast in Brazil. However, the use of bagasse used for this purpose will grow because estimates exist that 11,500 MW/h will be produced form sugarcane biomass by the year 2015 (Nascimento 2008). However, the potential to generate biofuels or useful energy is much more than is obtained to generate electricity, and use of this process alone means that a significant part of energy is wasted (Table 7).

The energy conversion efficiency, of actual ethanol production in Brazil, could be separated in three simulation models based in 1 ton of sugarcane stalk. The system used to convert biomass energy actually involves gasification-electricity generation, enzymatic hydrolysis, and pyrolysis Gasification-electricity generation is the more established technology for use of energy from bagasse (Hassuani et al. 2005). For this process, lignin has a higher heating value (24,471.2 GJ/ton dry weight), whereas the sum of cellulose, hemicelulose, and other extracts, all together, have a heating value of about 17 GJ/ton DM (Demirbas 2001). Sugarcane breeding for simultaneous production of sucrose and electricity must focus mainly in double purpose sugarcanes, with higher lignin and fiber content.

Evaluations by Hamelinck et al. (2005) conclude that the available technology at that time for enzymatic hydrolyzes of biomass, based mainly on dilute acid hydrolysis, has about 35% efficiency of conversion to ethanol. The overall efficiency, with electricity coproduced from the nonfermentable lignin, could be about 60%. Improvements in pretreatment and advances in biotechnology, especially through process combinations, can bring ethanol efficiency to 48% and the overall process efficiency to 68%. Additionally, at long term, the authors calculate that the efficiency of the fermentation of C5 sugars will increase dramatically, and more efficient pretreatments in the next 20 years will occur, with conversion yields possibly reaching 93%, much more than calculated for other alternative conversion systems.

Between the pretreatments, enzymatic hydrolysis generates more possibilities for the development of a more complex biorefinery complex associated with sugarcane or other lignocelluloses use as a source of energy. Looking at the US-DOE webpage (http://www1.eere.energy.gov/) one can see that by far the

Table 7 Show the energy present in distinct components of 1 ton sugarcane crop

Component yield	Energy (MJ/tc)
150 kg sugar	2,500
135 kg of stalk fiber	2,400
140 kg of leaf fiber	2,500
Total	7,400 (0.176 Toe)

MJ, Megajoule, equivalates to 3.6 kW; Tc, cubic ton; Toe, ton of oil equivalent

majority of funds are for grants in cellulosic ethanol and biorefineries. For this kind of technology, sugarcane breeding must focus on double purpose canes with lower lignin and S/G ratios, and more latter, when this technology will be more develped, the energy cane could be the major target. This means that in addition to increasing cane yield/ha, mitigating the negative effects of lignin in cellulosic ethanol production by or/and reduction of amount change lignin composition is an important goal. Ways to accomplish that goal could include reduction in guaiacyl content and/or increasing levels of molignols more easily degradable, (such as p-coumarate and p-hydroxybenzoate, hidroxycinnamic acid amides), reducing the feruloyl acid content, changing the patterns of lignin polymerization through alterations of the activity of monolignol-specific oxidases (peroxidases and laccases), and by reduction of the acetylation of lignin (Weng et al. 2008).

The third alternative, fast pyrolysis of bagasse offers the advantages of a liquid bio-oil product that can be used as potential fuel after modification, and also as a charcoal. For the biomass with higher cellulose content, the pyrolysis rate became faster, and ash could have negative values in the charcoal residue. The cellulose and lignin content in the biomasses could be important parameters to improving pyrolysis yield, but heir effects are still unknown (Gani and Naruse 2007). The bio-oil could find a market as a substitute for petroleum products, but some of its properties trigger additional costs. Sugarcane breeding for this energy conversion strategy will not take care of cell wall composition, but mainly in fiber content and cane yield/ha.

29 Conclusions

Breeding of sugarcane for production of energy from biomass depends mainly on the conversion technology that will be used. There are too many alternatives to conversion of biomass in ethanol or other more directly used forms of energy. All technologies are neither fully developed nor optimized. There is also the possibility of the integration of first and second generations of these technologies, but this possibility also depends on further technological improvements.

Overall, despite these technologies not being developed enough, the need of development of double purpose or energy cane is a high demanded task, considering the mean time of 12 years is needed to raise a new cultivar for each of these different purposes and the prevision that in 20 years we can reach the maximum efficiencies in energy conversion by enzymatic hydrolysis. This means crossing our modern hybrids with wild *Sacharum* species, such as *S. spontaneum*, and further process of nobilization. Because cell wall properties phenotypes characterization becomes crucial in these breeding efforts, we need to accomplish a complex analysis of plant cell wall structure in parallel to improvement efforts. Lignin and phenolics biosynthesis synthesis or degradation, and even structure (lignin) is far from well known, particularly in grasses.

Sugarcane genome sequence and functional genomics have a great potential to speed up this process, and changes introduced in cell wall by breeding or transformation will probably decrease resistance to biotic stress, a situation that underlines the need of use of transgenic approaches to be included could mitigate this potential problem. On the other hand, it is clear that cellulosic ethanol creates a rich source of bioproducts, and the concept of biorefineries associated to ethanol producing plants seems to be an obvious next development. The creation of a new industry on a large scale will require much basic and applied work on methods used to convert lignocellulose to value-added products, as well as new demands for sugarcane breeding, because several significant problems must be overcome to make the process ready for large-scale use. More breeders working in interdisciplinary research fields, not only in sugarcane but other biomass, seem to be a basic need to make renewable energy the major source of energy, helping to mitigate the climate changes that brings the risk of the feasibility of our planet survival.

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Chapter 14
Cell Wall Genomics in the Recombinogenic Moss *Physcomitrella patens*

Michael A. Lawton and Hemalatha Saidasan

1 Introduction

The growing interest in cellulosic ethanol as a sustainable biofuel has focused attention on modifying plants and plant cell walls in particular so that they can serve as a more effective feedstock for ethanol production. A substantial effort is now underway to develop methods that can increase the efficiency of cellulose breakdown by chemical or enzymatic pretreatments in order to generate fermentable sugars. This approach is leading to novel technologies for preprocessing and cell wall digestion, and the identification, selection, and production of enzymes with improved properties for industrial application (for example, heat stable hydrolytic enzymes).

Another extremely promising approach involves directly modifying the structure of the plant cell wall so that cellulose present in the biofuel feedstock is more readily available for downstream processing and is more easily hydrolyzed to fermentable sugars. A major challenge for biologists is to make these modifications without compromising the normal growth, function, and health of the plant. For example, efforts to reduce lignin content and thereby increase the accessibility of cellulose microfibrils to enzymic hydrolysis might also render the native plant more susceptible to infection by necrotrophic pathogens or compromise the ability of the plant to withstand physical and abiotic stress. While the plant cell wall is a primary focus for improvement, the economics of agriculture and the potential competition with food crops for arable land demands that we make every effort to improve the overall efficiency of biofuel crops and focus also on traits that increase yield, reduce losses to disease, decrease the need for inputs such as fertilizer and water, and allow biofuel crops to be grown on land that is currently underutilized or is unsuitable for conventional agriculture. For example, engineered or selected drought-tolerant

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biofuel crops might be grown in drought-prone regions or on lands that have poor soils that may not be able to sustain the cultivation of food crops.

If we are to manipulate the plant cell wall in a predictive and useful manner, we need to develop a better understanding of the genetic, molecular, and biochemical mechanisms involved in cell wall biogenesis and maturation. This will require a more complete understanding of how plant cell walls are constructed at the molecular and biochemical level. In particular, we need to develop a broader and deeper knowledge of: (1) the nature of the primary and secondary structural components associated with different types of plant cell walls (the major carbohydrate and phenylpropanoid-derived polymers as well as structural proteins); (2) the chemical interactions of these components with each other via covalent and noncovalent modifications; (3) an understanding of the spatial arrangements and sub-domains of different cell wall components (for example, in primary and secondary cell walls or in apical vs. lateral regions of plant cells in a file of cells); (4) the nature and regulation of the enzyme activities responsible for the synthesis, deposition, modification, and degradation of cell wall components. By studying these features of cell walls in a variety of disparate cell types (for example, in cells of different tissues, different developmental stages or from highly specialized cells present in particular species or organs), and under a range of different environmental conditions, (such as nutrient limitation, environmental stress, disease, and physical stress), we will be able to build up a picture of the functions of the different cell wall components, how they interact and how they are regulated. For example, the walls produced by leaf epidermal cells of tobacco are likely to be substantially different and have different functions than the cell walls present in cotton fibers, in the casperian strip or in cells undergoing xylogenesis. By comparing these different tissues and species, we will gain a deeper understanding of how nature is able to build and modify specific types of plant cell walls to produce cells with distinct structural, chemical, and biochemical properties. This is the goal and promise of synthetic biology: the rational design and construction of biologically based organisms. Its execution will require a substantial effort in the fields of genomics and phenomics.

Genomic methods allow the genetic basis of cell wall variation within breeding plant populations (such as populations of inbred lines) to be assessed, genetically mapped, and, eventually, characterized at the level of DNA sequence. Molecular genetics can complement this natural variation, and allow novel variants of plant cell walls to be generated by the targeted modification of specific genes. These approaches allow specific modifications in the structure of the plant cell wall to be linked to the presence and activity of particular genes or alleles of gene and can be applied to well-studied model systems as well as emerging systems. These genetic tools, which offer a precision not available through other methods (such as the use of chemical inhibitors or growth regulators) promise to contribute significantly to our understanding of how individual plant cell wall types are specified and assembled. The relationship between genes and the structure of the plant cell wall forms a technological platform for understanding and designing plant cell walls with specific desirable properties. This chapter examines the utility of the recombinogenic moss *Physcomitrella patens,* which

is emerging as a useful and complementary system to higher plants for examining gene function and for understanding fundamental processes in plant biology. The potential of this system to advance gene discovery and functional validation in cell wall biology will be emphasized.

2 Identifying Genes and Gene Function

For geneticists, the question "How are plant cell walls constructed?" is usefully rephrased as "Which genes are involved in the biogenesis and modification of plant cell walls?" Several complementary technologies can address this question and help us understand the relationship between genetic makeup and traits related to the plant cell wall. Genetic association studies allow the presence of particular genetic loci (e.g., QTLs, genes or alleles) to be correlated with the expression of individual traits, such as high sucrose accumulation, reduced lignin content, and so on (for a more extensive discussion of the application of this approach in sugarcane, see the chapter 13 and Papini-Terzi et al., 2009). Association studies can be conducted by following the segregation of specific alleles or physical gene markers, such as SNPs, microsatellites or AFLPs, with the expression of a particular trait of interest in the offspring of a genetic cross. Association studies can also be used to define the temporal and spatial pattern of expression of particular genes and thereby flag candidate genes that may contribute to the trait of interest, for example during secondary thickening of cell walls. A major advantage of such studies is that they can be performed directly in the target crop of interest (such as sugarcane, or any of the other grasses and plants being considered as cellulosic biofuels) and will likely identify genes that are directly relevant for trait enhancement in the crop itself. These approaches have benefited greatly from the application of genomic tools, including the development of large collections of expressed sequence tags (ESTs); the use of microarrays and other methods for gene expression profiling, whole genome sequencing; and (in the case of the grasses) the extensive synteny among genomes of different species which facilitates the translation of results obtained in one system to another (Bennetzen and Freeling 1997). The application of molecular methods can also assist traditional breeding programs through marker-assisted selection. It is likely, as additional genomes are sequenced, that we will also see the widespread use of whole-genome sequencing methods, including genome resequencing of segregating populations of plants, to assist and guide breeding programs and help define and characterize genes, and loci (such as QTLs) that contribute to useful traits.

One limitation of association studies is that it is restricted to traits and genes present in the breeding population of the target crop plant. While this may not present a problem for traits for which there is considerable variation in natural and cultivated populations, it can be a major hurdle for some crops and for certain traits, such as disease resistance, where there may simply be no useful germplasm to work with in either the cultivated or wild varieties of a plant. A further limitation is that it can

take considerable effort and resources to establish the breeding populations (such as near-isogenic and recombinant inbred lines) that are needed to develop markers that cosegregate with specific traits (and genes) of interest. Some properties that may help develop biofuel crops, such as the ability of plants to degrade their own cell walls, may involve the introduction of genes from other organisms, such as saprophytic or pathogenic microbes. This approach is likely to require considerable optimization, the use of many native and engineered candidate genes and the generation of a large number of transgenic plants (see the chapter 12 in this volume by Lam et al.). Generating these plants can be problematic for some biofuel crops, such as sugarcane or other grasses, for which transformation is currently relatively inefficient. In such cases, it is useful to test the candidate genes in a simpler and more experimentally amenable format.

One approach to overcoming these problems is to work with model systems that are more genetically tractable or for which greater genomic and genetic resources are available. An elegant example is maize, which has highly developed genetic and molecular genetic resources and offers the possibility of isolating single genes associated with individual traits through classical mapping and gene walking techniques as well as through the use of transposon tagging strategies. Alternatively, one can make use of models such as *Arabidopsis thaliana*, which allows genes to be routinely isolated by positional cloning and has rich genomic, genetic, and bioinformatic resources. Although the use of models is attractive, one must be aware of the limitations of each system, and in particular whether the model system displays the appropriate biological features of interest and whether results obtained from the model (in the form of knowledge, mechanisms, identification of genes, proteins, and small molecules) are translatable to the target crop.

2.1 Approaches for Forward and Reverse Genetics

Forward genetics makes use of natural variants or artificially generated mutants and hones in on the loci and genes of interest. Such approaches can be complemented by reverse genetic methods, in which genes of unknown or suspected function are functionally interrogated through their artificial manipulation. Reverse genetics can be used to investigate the contribution of genes to particular phenotypes in the target plant species. It can also be used to assay novel versions of native genes that may encode a protein with different biochemical properties. The importance of reverse genetics has increased as our ability to physically access genes has increased, especially in the form of completed genome sequences. In short, there are large numbers of gene sequences available in the completed genomic sequences of both angiosperms and lower plants, and a large proportion of these genes are of unknown function. This trend is likely to continue as the cost of whole genome sequencing falls. However, our understanding of the functions of individual genes and gene networks has lagged behind our ability to sequence and catalog genes, and there is a pressing need for methods that can speed up the identification of gene function.

A number of methods are currently widely used to define gene function and the function of their encoded products. These include gene overexpression in a heterologous system followed by biochemical assay of the purified gene product; overexpression of genes in the native (wild type) host, which is potentially useful for genes that exert a quantitative effect on phenotype; the expression of genes that express a tagged protein for subcellular visualization in vivo or for the purification of native protein complexes; the expression of mutants that confer gain-of-function phenotypes on transgenic plants (including mutants generated by rationally designed approaches or by directed evolution); stable suppression of endogenous genes by antisense RNA, RNAi, and related technologies (Petsch et al., 2010); transient suppression of gene function by virus-induced gene silencing (VIGS) (Burch-Smith et al. 2004); and the inactivation of endogenous genes by transposon insertion (Walbot 2000) or the integration of T-DNA elements (Parinov and Sundaresan 2000). A collection of gene knockouts has been created in Arabidopsis through the random insertion of *Agrobacterium tumefaciens* T-DNA into the genome followed by sequencing of regions flanking the T-DNA elements (Alonso et al. 2003). This approach is effective for small, defined (sequenced and annotated) genomes and provides a common pool from which knockouts can be found and used for each and every gene in the genome. For larger genomes, for genomes not yet fully sequenced and annotated, and (in particular) for genomes in which there is considerable genetic redundancy, such as polyploid grasses like sugarcane, these approaches may be of limited value since it is may not be feasible to fully cover the genome with mutagenic T-DNA elements, or to sample a sufficient number of individuals to ensure complete coverage (mutation) of the genome. The presence of gene families or the operation of other mechanisms that result in functional redundancy can complicate these approaches, and the effects of a many single gene-insertion events may be silent or minimal. The example of maize shows that the problem of mutagenizing a large genome with an insertional element can be overcome by using very active transposable elements, such as Ac/Ds and Spm in a diploid background. The heterologous expression of these mutagenic elements in diploid varieties of sugarcane and other biofuel grasses may be a useful approach to help define useful traits in those systems. The advent of methods for gene suppression using RNAi has been extremely useful for creating pseudo-knockouts of target genes. The major limitations of RNAi are that it may not completely suppress gene expression (although this can be an advantage for essential genes) and may lead to the suppression of additional, unintended targets within the genome. This is more likely to be a problem in large genomes that are not well characterized. A recent development that offers great promise is the use of zinc finger nucleases that can be targeted to cleave specific loci via a short guide nucleic acid (Durai et al. 2005).

All these methods rely on transformation to deliver transgenes, whether they are designed to overexpress a native or foreign gene or to express an RNAi construct. Consequently plants for which transformation is inefficient are at a disadvantage and in these systems it will be difficult and expensive to generate transgenic lines to validate gene function of candidate genes, let alone the use of transformation to discover gene function in a less-directed, serendipitous manner. Such approaches require the use of plants for which highly efficient transformation is available,

whose genome is characterized and which are amenable to genetic manipulation. The moss, *P. patens*, represents such a system and has considerable potential as a research tool and companion system for understanding the genetic basis for plant cell wall biogenesis and function (Lee et al. 2005b). This system can be used for de novo gene discovery and as a rapid gene assay system to test the contributions of genes that have been flagged from research in other systems (for example from whole genome association studies performed in grasses).

3 Functional Genomics in *Physcomitrella patens*

The moss, *P. patens*, is an emerging system for functional genomics and, in particular, for studies that require precise, predictable, and targeted genome manipulation (Box 1) (Schaefer and Zrÿd 2001; Reski 2003; Lee et al. 2005a; Cove et al. 2006). The principal attraction of this system is its ability to undergo high recombination with an efficiency that is similar to that observed for *Saccharomyces cerevisiae* and at least two orders of magnitude greater than that is found for angiosperms (Fig. 1).

Gene disruption is performed by creating a construct in which a selectable marker gene is flanked by two DNA sequences derived from the target gene. Constructs are then introduced into *Physcomitrella* protoplasts using a PEG/heat shock method, and the resulting transformants are selected for their ability to grow in the presence of an antibiotic. The rate of homologous integration of these constructs is orders of magnitude higher than random integration, and this makes it relatively easy to recover cells that have undergone gene disruption and knockout. Efficient integration at target sites within the genome means that it is simple to construct mutants for any *Physcomitrella* gene and recover gene knockouts from a relatively small population of initial transformants. Because the haploid phase is predominant, the effects of gene knockouts are seen immediately in dividing transformed protoplasts. In addition to its use for creating gene knockouts, the ability to

Box 1 Experimental Features of *Physcomitrella patens*

- Bryophyte, member of the *Funariales*
- Easily cultured on solid medium; suitable for small-scale and high-throughput formats and screens
- Well suited to cell biological studies – most tissues only 1-cell thick
- Haploid genome, 27 chromosomes, ~511Mbp
- Genome fully sequenced, annotated; Large EST collections
- Easily transformed by PEG/heat shock, DNA direct uptake
- Highly efficient gene targeting allowing for gene knockout, *in situ* mutation and delivery of transgenes to specific chromosomal loci

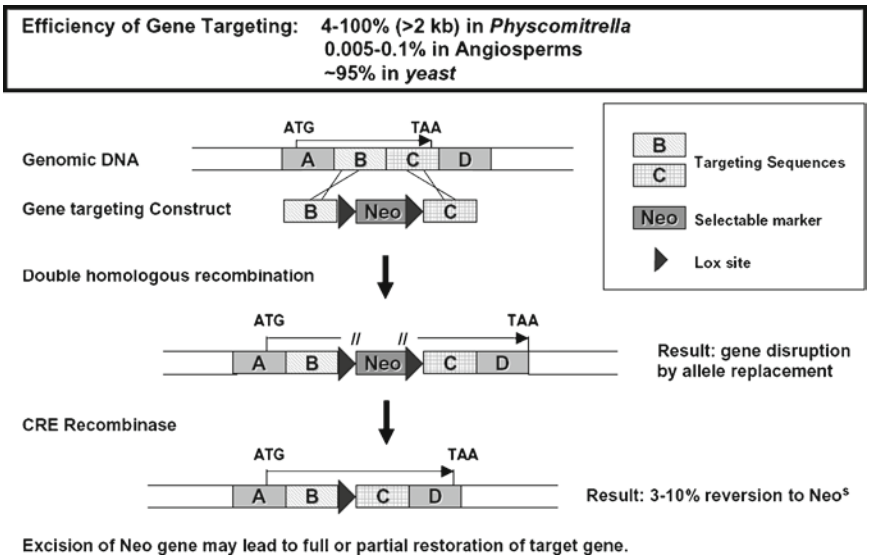

Fig. 1 Creating gene knockouts in *Physcomitrella patens*. Gene knockouts are constructed by placing two internal but discontinuous regions of the target gene (B and C) on either side of a selectable marker gene, such as the one encoding neomycin resistance (Neo, in the figure). This construct is introduced in *Physcomitrella* protoplasts using standard PEG/heat shock methods, and the resulting cells plated out on selectable medium. Plant colonies derived from cells that undergo a double homologous recombination event can be screened using PCR for the presence of the predicted junction between the selectable marker gene and native flanking gene sequences. The result is the replacement of the internal region of the target gene with the selectable marker and the consequent loss of function of the target gene. Subsequent transient expression of CRE recombinase removes the marker gene from the genome and (depending on the excision event) may allow the target gene to become functional once more

manipulate the *Physcomitrella* genome makes it an ideal system for protein engineering, since wild type genes can be replaced at the native genomic locus with in vitro-generated site-specific mutants.

The utility of this system has been substantially enhanced by the completion and annotation of the *Physcomitrella* genome sequence (Rensing et al. 2008), along with the characterization of EST collections (Rensing et al. 2002). Genome information and annotation is extremely useful for selecting candidate genes for targeted knockout and mutation in *Physcomitrella*. First, it provides a snapshot of the biosynthetic pathways and activities present in *Physcomitrella*, and the degree to which these are conserved with, or distinct from those found in higher plants. Second, genome annotation allows the complexity of genes families to be determined. This is of practical significance since it is easier to address the function of genes that are present as single or low copy families. *Physcomitrella* is particularly useful for functional genomic studies where it contains only one or a few members of a gene family that is present in larger numbers in crop plants. In such cases, knockout of one or two genes in *Physcomitrella* can provide important information

on the basic function of the entire gene family without having to create a knockout line for multiple genes. These features have contributed to a growing interest in using *Physcomitrella* as a companion system for studies of crop plants (which are principally seed-bearing plants).

4 *Physcomitrella* Growth and Development

Following germination, *Physcomitrella* spores undergo polar growth, divide and give rise to files of filamentous protonemal cells which form side branch initials which also undergo polar tip growth (Cove 2000; Pressel et al. 2008). Repetition of this pattern gives rise to a highly branched filamentous colony which continues to grow under favorable environmental and nutrient conditions. Buds develop in distal protonemal cells and give rise to the gametophore tissue, which consists of leaflets arranged in a phyllotaxic manner, and which, in turn, produce the gamete-bearing archegonia and antheridia, and (after fertilization), the diploid sporophyte, which is much reduced compared to higher plants. Cellular ablation studies indicate that leaflet development in *Physcomitrella* reflects the operation of locally acting cues rather than the signals that act over long distances (Harrison et al. 2009). The cells in the leaflets of the gametophyte are arranged in a plane that is one-cell thick, save for the midrib. This makes observation very easy and *Physcomitrella* a superb system for in vivo imaging and cell biology. Although *Physcomitrella* is a nonvascular plant and lacks the highly differentiated xylem and phloem tissues characteristic of angiosperms (Hebant 1977), it does contain additional specialized cells, called hydroids, which conduct water, and presumably nutrients too, in gametophore tissues. These fundamental features of moss architecture and tissue differentiation mean that mosses such as *Physcomitrella* cannot be used to study cell wall differentiation associated with highly specialized forms of cell wall modification such as xylogenesis, or cell plate formation in phloem. Rather, its utility lies in its potential as a system for studying the construction of the primary plant cell wall and highly conserved secondary modifications.

The prominence of polar tip growth in *Physcomitrella* provides an interesting opportunity for studying plant cell wall biology. In the protonemal tissue, the individual components responsible for cell wall biogenesis, modification, and maturation are elaborated over space and time, with the newest part of the cell wall being continuously generated at the growing tip, and with maturation taking place in cells subtended by the growing apical region. The apical region of the protonemal tip cell has a minimal cell wall that is nonetheless strong enough to provide cellular integrity and counter the turgor pressure of the protoplast. At the same time, this region must continually undergo fusion with secretory vesicles that deliver new material to the growing cell wall. These features make the apical tip cell an excellent choice for examining mechanisms involved in vesicle trafficking and the delivery of new materials to the growing cell wall, while regions adjacent to the growing tip would

be expected to be highly active in processes that control extension growth, including the controlled slippage of cellulose microfibrils. Similarly, sub-apical cells would be expected to undergo secondary modifications and be more refractive to digestion with hydrolytic enzymes. This is supported by exposure to hydrolytic enzymes such as driselase, which preferentially releases protoplasts from the tip of the apical cell, while older protonemal cells remain more refractory to cell wall digestion. This observation suggests that the cell walls of mature protonema and gametophore tissues (which are also relatively more resistant to enzymatic digestion with driselase) are strengthened through the deposition of novel cell wall components that are not hydrolyzable by the enzymes in the driselase extract or that have become refractory to digestion through the introduction of cross-links or other modifications within existing wall polymers. Such modifications are of great interest as they may reveal alternative approaches and mechanisms (compared to seed plants) for strengthening the plant cell wall while simultaneously permitting cellular elongation and growth.

The spatial separation of the components of cell wall biogenesis in growing protonemal cell files must also be accompanied by the appropriate spatial and temporal regulation of genes, proteins and enzymes involved in the synthesis, delivery, or deposition of cell wall molecules. The synthesis of these components must be coordinated and regulated so that they occur in a manner that is in concert with the developmental needs of the growing protonemal filament. Given the spatial separation of development in a file of protonemal cells, we might expect mutants that affect different stages of cell wall biogenesis to generate phenotypes that are apparent in cell types of different ages and that represent different developmental stages and fates. For example, mutants that affect cell wall secondary thickening may alter the susceptibility of sub-apical cells to digestion with lytic enzymes, while mutants that affect the loosening of existing cell walls may be compromised in their ability to form side-branch initials. Although polar cell growth is not a major feature of higher plants, it is present in pollen tube germination and associated with the asymmetric cell expansion characteristic of root hair cells and leaf trichomes. Growth and development of the gametophore more closely resemble the program observed in higher plants, with a meristem that continually elaborates new organs (first, leaves and then the terminal reproductive organs). In these tissues, cell wall biogenesis occurs in a more isometric fashion, and modification and secondary thickening will occur more uniformly, as it does in the cell walls of angiosperms.

5 Features of the *Physcomitrella* Plant Cell Wall

The advantages of *Physcomitrella* for studying the plant cell wall have been noted by others (Lee et al. 2005a). A number of studies that focus on the *Physcomitrella* cell wall and the function of cell wall-related genes have been published in the last few years and this is likely to increase as this utility of the system for genomics is

combined with an increasing knowledge of the structural features and properties of the *Physcomitrella* cell wall. The utility of *Physcomitrella* for advancing cell wall biology was demonstrated in an elegant study by Lee et al., who showed, through the creation of gene knockouts, that specific members of the arabinogalactan proteins (AGPs) are involved in apical cell tip growth (Lee et al. 2005b). In addition to taking advantage of the molecular genetic properties of *Physcomitrella*, this study also illustrates one underrated advantage of the system, which is that the particular biology of the plant (in this case the readily scored phenotype of polar tip growth) provides an opportunity to observe phenotypes that may not be as readily apparent in higher plants. This may reflect the fact that certain biological phenomena, such as the rate of polar cell tip growth or the rate of cell division (which would be reflected in cell length in protonemal files), are simply exquisitely sensitive to genetic perturbations, and cumulative effects of even small quantitative differences can be readily observed over time. In some cases, the ability to observe a phenotype for a gene knockout in *Physcomitrella* may reflect the presence of a single gene or smaller gene family in the moss, compared to higher plants. Alternatively, *Physcomitrella* may have fewer compensatory mechanisms compared to higher plants, allowing the effects of single gene mutations to be clearly observed. In such cases, the corresponding gene disruption in *Physcomitrella* may give rise to an observable or quantifiable phenotype that would not be apparent in higher plants (Table 1).

Carbohydrate chemistry and related analytical methods have shown that *Physcomitrella* cell walls contain many of the major structural components present in angiosperms (Sarkar et al. 2009). These include the major carbohydrate molecules, cellulose, xyloglucans (XGs), pectin (including the oligosaccharides rhamnogalacturonan II [RGII]), and mannans, which are more abundant in *Physcomitrella* than in higher plants. *Physcomitrella* also contains important structural proteins and enzymes such as AGPs, peroxidises, expansins, and XETS. Notably absent is lignin, although mosses do produce XGs (Popper and Fry 2003; Pena et al. 2008). Mosses can produce UV-absorbing phenolic compounds (Clarke and Robinson 2008), which may interact with and cross-link to other cell wall components. We have recently observed that the regulation of phenolic compound levels in *Physcomitrella* is very dynamic, and that the production of these compounds can be induced in response to stress and to physical damage. Interestingly, production of these compounds is also up-regulated when the production of other major cell wall components is suppressed, implying the presence of compensatory regulatory mechanisms that control the accumulation of different cell wall components (Ucumzu and Saidasan, personal communication).

Cellulose is readily detected in *Physcomitrella* cell walls by staining with calcofluor white (Lee et al. 2005b) and has also been detected using cross-reacting monoclonal antibodies (Moller et al. 2007). Subsequent studies have shown the presence of β(1→4)Gluc linkages in hydrolysates of the *Physcomitrella* cell wall, consistent with the presence of cellulose. Recently, atomic force microscopy was used to demonstrate the presence of *Physcomitrella* cell wall fibers whose alignment and distribution are consistent with that expected for cellulose microfibrils (Wyatt et al. 2008). The recent completion of the *Physcomitrella* genome sequence, coupled with a

Table 1 Major features of the Physcomitrella cell wall

Cell Wall Components	Principal Features	Methods	Comments	References
Cellulose	Major polymer of plant cell walls. Presence of microfibrils in *P. patens* revealed by atomic force microscopy	Staining with calcafluor white, linkage analysis, mAbs MC CBM3a,	Presumed to be deposited at the cell wall by typical rosette structures	Roberts and Bushoven, 2007, Liepman et al., 2007
CESA genes	Encode cellulose synthases	Informatic analysis of the *P. patens* genome	Synthesize cellulose as *trans*-membrane rosettes	Roberts and Bushoven, 2007
CES-Like (CSL) gene family	CesA-like genes: CSLs A, C, D present. CSLs B, E, F, G, H absent	Bioinformatic analysis of ESTs and sequenced *P. patens* genome	Responsible for synthesis of non-cellulosic cell wall polysaccharide backbone	Roberts and Bushoven, 2007, Liepman et al., 2007
CSLA	Encodes mannan synthase	Heterologous expression in *Drosophila* S2 cells, assay of enzyme *in vitro*	Mannans appear to be more abundant in *P. Patens* and other mosses, than in angiosperms	Roberts and Bushoven, 2007, Liepman et al., 2007
Mannans	β (1→4) linkage comprising mannan is present in Physcomitrella	mAbs, TLC, HPLC of driselase-digest products. mAb BS 400-30	Present in chloronmal and caulonemal tissues, cell junctions and buds	Liepman et al, 2007, Popper and Fry, 2003# Moller et al., 2007
Callose	Expressed in spores. Induced at wound sites	Aniline Blue Staining	Callose synthase homologs present as 12-copy family of *PpCalS* genes	Schuette et al, 2009, M. Lawton, unpublished data
β(1→3) glucan	Major linkage in callose	mAb BS 400-2		Moller et al., 2007
β(1→4) galactan		mAb LM5	Identified by CoMPP (Comprehensive Microarray Polymer Profiling)	Moller et al., 2007
Pectin/ Homogalacturonan (HG)	HG is a linear homopolymer of (1→4)-α-linked-d-galacturonic acid (GalA)	mAbs JIM5, 7, 2F4 Homology with Arabidopsis genes	Methyl esterified HG present in sporophyte-rich fraction. Galacturonosyltransferase (GAUT)-like homologs (required for pectin synthesis) are present in *P. patens*	Moller et al., 2007 Sørensen et al., Sterling et al., 2006. Bacic, 2006

(continued)

Table 1 (continued)

Cell Wall Components	Principal Features	Methods	Comments	References
Rhamnogalacturonan II	Modified HG (pectin). Involved in borate cross-linking. Found in several moss species	SEC/ICP-MS ESI-MS GC/EI-MS 11B-NMR	Proposed as evolutionarily early cross-linking network. Rhamnose biosynthetic enzyme I identified as drought stress protein in *P. patens*	Matsunaga et al., 2004 Kenrick, 2000 Wang et al., 2009
β(1→4) xylan		mAb LM10		Moller et al., 2007
Xyloglucans (XG) XG + Fucose	*P. patens* contains XXGGG as opposed to the XXXG form found in angiosperms	NMR, MALDI-TOF, ESMS, linkage analysis, HPLC, TLC. mAb LM15, CCRC-M1	XGs are the major hemicellulosic component of angiosperm primary cell walls	Peña et al., 2008 Popper and Fry, 2003# Moller et al., 2007
Xylans	Unsubstituted and low-substituted xyloglucans	mAbs LM10, LM11	No signal detected in bryophytes, indicating absence of unsubstituted XGs	Carafa et al., 2005#
β(1→4) arabinan		mAb LM6		Moller et al., 2007
AGPs	*P. patens* AGPS contain the unusual terminal sugar 3-*O*-Me-L-rhamnose	Crossed electrophoresis, GLC, mAbs CCRC-M7, JIM 13, LM2, Mac207	CCRC-M7 labels water conducting cells in several hepatics	Fu et al., 2007, Ligrone et al., 2002#, Moller et al., 2007 Lee et al., 2005
XET	Involved in cell wall reorganization. Hydrolyze and rejoin XGs during slippage of adjacent cellulose microfibrils	XET test paper activity stain	XET activity found at sites of growth and cell elongation	Van Sandt et al., 2007
Expansins	Similar number of genes as in Arabidopsis, lacking EXLA, EXLB	Informatic analysis of JGI genome sequence	Involved in pH-dependent growth; weaken links between cellulose microfibrils	Carey and Cosgrove, 2007 Schipper et al., 2002, Li et al., 2002
Germins	Cell wall localized oxalate oxidase activity; converts oxalate to hydrogen peroxide	ESTs identified by homology; Mn-SOD purified from *P. patens* cell wall	PpGLP6 encodes manganese-containing extracellular superoxide dismutase activity (Mn-SOD)	Nakata et al., 2004,

Phenolics	Induced by wounding Induced by UV and serve as UV protectants in arctic mosses	*In situ* staining Spectroscopic analysis	No evidence for material that is structurally similar to lignin of tracheophytes. However, soluble phenolics may become cross-linked	Lawton, unpublished data Clarke and Robinson#
Peroxidases			Involved in response to pathogens	Lehtonen et al., 2009
HRGPs/Extensin	Major repetitive protein in higher plants HRGPs	mAb JIM20 BLASTsearch	mAb JIM20 shows cross-reactivity. No clear homologues in genome.	Moller et al., 2008M. Lawton, unpublished
N-glycans	Universally present in plants, synthesized in the Golgi		Gn2M3FX core present in Physcomitrella. Physcomitrella knockouts of fucosylteransferase and xylosyltransferase grow normally	Mega, 2007, Koprivova, et al., 2004, Viëtor et al.,

[#]Studies made using bryophytes other than *P. patens* but included for comparison.
mAb: monoclonal antibody

detailed informatic analysis, has greatly aided our understanding of the machinery of cellulose biosynthesis in *Physcomitrella*. These studies have revealed that *Physcomitrella* contains several genes related to the *CESA* gene family of cellulose synthases that are present in higher plants (Liepman et al. 2007; Roberts and Bushoven 2007). These genes are likely to encode the principal machinery of cellulose biosynthesis, and their functional characterization and manipulation are of great interest. The creation of gene knockouts of individual or combinations of *CESA* genes should help us understand the specific functions and sites of action of individual *CESA* gene members. Such mutants may also be useful for functional assays of *CESA* genes from other plants, which can be assayed for their ability to complement the phenotype of knockout plants or used to alter the cellulose synthesizing capabilities of the moss. This approach may also be exploited to assay modified cellulose synthases with altered or novel biochemical or regulatory properties.

The *CESA*-like (*CESL*) genes are structurally related to cellulose synthases but encode enzymatically distinct glycan synthases, which are responsible for the biosynthesis of other, noncellulosic polymers (Liepman et al. 2007; Roberts and Bushoven 2007). Comparison with angiosperms reveals that while *Physcomitrella* encodes members of the CSL-A, C and D families, the CSL-B, E, F, and G families are absent. The CESL-A protein family is involved in mannan synthesis, and the presence of mannans in the *Physcomitrella* cell wall has been confirmed by linkage analysis and related analytical methods, as well as by the use of specific monoclonal antibodies. The ability of the *Physcomitrella CSLA1* and *CSLA2* genes to synthesize mannans in vitro was also demonstrated by heterologous expression in insect cells (Liepman et al. 2007). *Physcomitrella*, like higher plants, also synthesizes β(1→3) glucans in the form of callose, which can be detected in spores, as well as in injured tissues where it presumable acts to seal off wound sites (Chi-Tai Tang, personal communication).

XGs, the major hemicellulosic component of seed plants, have been identified and characterized in *Physcomitrella*. *Physcomitrella* XGs are structurally distinct from those found in most higher plants, which produce XXGGG-type molecules, although the grasses, as well as some Lamids, do produce XXGG and XXGGG-type XGs (Gibeaut et al. 2004; Hoffman et al. 2005; Pena et al. 2008). *Physcomitrella* XGs are unusual in that they contain galactosyluronic acid and branched xylosyl residues, and it has been suggested by Peña et al., that these side-chains, which increase water solubility, may assist in the transport of the newly synthesized XG from the Golgi to the cell wall and may also reduce the ability of XGs to interact with each other and with cellulose microfibrils. These properties, conferred by the XG side-chains may compensate for the absence of *O*-acetylation in *Physcomitrella* XGs, which is a feature of XXGGG molecules present in the *Poaceae*. It is clear from these comparative studies that plants as a whole are capable of manufacturing structurally diverse XGs. The ability to modify the *Physcomitrella* genome provides an opportunity to explore the genetic basis for generating structurally distinct XGs, including the modification of the primary backbone structure and the introduction of novel side-chain substitutions.

Several cell wall proteins have been characterized in *Physcomitrella*, including the aforementioned AGPs, which in addition to their role in tip growth are also

found associated with the water conducting vessels of liverworts. AGPs exist as a gene family in *Physcomitrella*, and it is likely that additional biological role for these molecules will be revealed as additional gene knockouts are constructed. In contrast to the AGPs, there are apparently no homologues in the *Physcomitrella* genome for HRPGs or GRPs, and therefore these wall proteins may represent more recent innovations in plant cell wall biology. Interestingly, monoclonal antibodies raised against higher plant HRPGs do detect cross-reacting material in *Physcomitrella* (Moller et al. 2008), but their apparent absence from the genome suggests that this may be due to cross-reactivity with other wall components. This raises a note of caution when interpreting the results obtained using heterologous antibodies to study the *Physcomitrella* cell wall. Nonetheless, the approach of using a panel of monoclonal antibodies, termed Comprehensive Microarray Polymer Profiling (analogous to the use of DNA microarrays to study gene expression) to probe the epitopic landscape of the *Physcomitrella* cell wall is an elegant and informative one and has largely been validated by chemical analysis and by data mining of the genome.

The principal biochemical mechanisms responsible for cell wall elongation appear to be conserved between *Physcomitrella* and higher plants, as judged by the presence of genes encoding XETs (Van Sandt et al. 2007) and expansins (Li et al. 2002; Schipper et al. 2002; Carey and Cosgrove 2007), which have been implicated in the reversible hydrolysis of cross-linking hemi-celluloses and the controlled slippage of adjacent cellulose microfibrils. As observed with the AGPs, we might expect knockouts in these genes to have an effect on elongation of the protonema, and this may prove a very useful assay system for studying the properties of this gene family, including the various isogenes that are present in potential biofuel crop species.

The other major cell wall component in *Physcomitrella* is pectin, whose presence is confirmed and implied by a variety of structural and chemical methods, by the use of monoclonal antibodies and by the presence in the genome of homologues of galacturonosyltransferase-like gene homologs, which are required for pectin synthesis. Interestingly, *Physcomitrella* contains modified pectins in the form of RGII (Kenrick 2000; Matsunaga et al. 2004), which is also present in higher plant cell walls, which contributes to cell wall strength through borate cross-linking. Rhamnose biosynthetic enzyme 1, which is involved in the biosynthesis of this molecule, has been found in *Physcomitrella* using a proteomics approach, where it was identified as a drought stress response protein (Wang et al. 2009). It has been proposed that RGII cross-linking may represent a primitive system for cell wall strengthening that predates the development of more sophisticated mechanisms associated with lignin biosynthesis and deposition (Matsunaga et al. 2004).

6 Target Identification and Functional Assay

Genes can be identified for knockout studies or in situ modification from a variety of sources and using a broad range of approaches (Fig. 2).

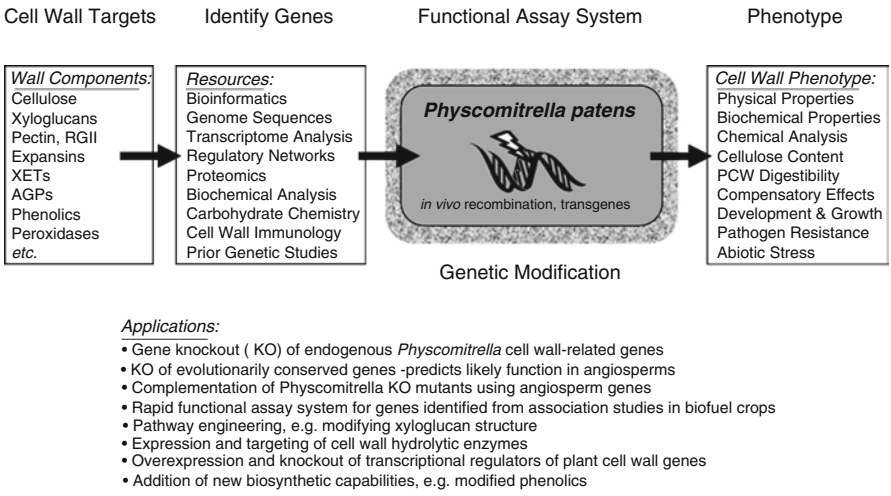

Fig. 2 Target Identification and Functional Assay. The scheme illustrates how genes may be selected for functional studies in *Physcomitrella*. Structural and molecular studies in biofuel crops as well as in *Physcomitrella* are coupled with bioinformatic analyses to select genes and design knockout constructs. For genes that are conserved with crop plants, the *Physcomitrella* gene homolog can serve as a stand-in to inquire about the general function of the corresponding gene in crop plant cell wall biogenesis. Knockouts create a basal system for functional complementation studies using crop plant genes, as well as an experimental platform for modifying the cell wall through the expression of mutant genes or natural genes that may confer novel properties (for example, novel carbohydrate linkages). *Physcomitrella* is also useful for ectopic transgene expression and the functional assessment of lead genes identified by genetic association studies in crop plants. The phenotypic consequences of genetic modification can be assessed using a variety of physical, biochemical and biological criteria and assays

In some cases, the likely function target is known to a first degree, based on studies in other systems. For example, the *CES*/*CSL* gene family forms a natural group of interest to the investigator, based on their identification as cellulose synthases and related glycan sysnthases in other plants (Liepman et al. 2007; Roberts and Bushoven 2007; Yin et al. 2009). The ability to create gene knockouts in *Physcomitrella* allows the contribution of these genes to cell wall biogenesis and architecture to be systematically examined. Moreover, it is relatively easy to construct double and multiple gene knockouts, since related genes can be replaced simultaneously using constructs that target conserved sequences, while selectable marker genes that carry LOX-sites can be recycled and reused in serial transformation following their excision by transient expression of the CRE recombinase (Fig. 1) (Schaefer and Zryd 1997). A large number of genes of interest are emerging from genetic linkage association studies, as well as from transcriptomic approaches such as DNA microarrays, SAGE, and digital Northerns based on high throughput cDNA sequencing. Again, *Physcomitrella* offers a simple and rapid opportunity to assess the contribution of these genes to the generation and function of the plant cell wall. In addition to helping delineate the role of genes of unknown function, the creation of knockouts in *Physcomitrella* also provides an opportunity to comple-

ment function with genes from higher plants, including those from biofuel crops. This can be helpful for plants such as sugarcane, where the presence of multigene families, along with the presence of multiple genomes, can make it difficult to determine which member of a gene family is responsible for a particular trait or biochemical activity. Finally, *Physcomitrella* also provides an excellent and predictable system for the ectopic expression of foreign plant proteins, especially those that require plant-specific posttranslational modification or delivery to a plant-specific sub-cellular compartment, such as the chloroplast or plant cell wall.

While it is easy to manipulate the genome and create gene knockouts in *Physcomitrella*, the development of functional assays lags behind (this is also true in higher plants). Clearly, there is a need for a better physical description of the *Physcomitrella* cell wall so that the effects of specific gene modifications can be characterized at the chemical level (exemplified, for example, by the loss of a particular sugar or linkage, or the absence of cell wall proteins or enzymes). The development of panels of monoclonal antibodies should aid in the characterization of cell wall modifications (Moller et al. 2007; Moller et al. 2008), and it is of note that the antibody reagents developed for screening cell wall features in Arabidopsis are also able to cross-react with their counterparts in the cell walls of *Physcomitrella*. Similarly, even simple stains can be quite informative for examining gross changes in cell wall composition and structure. While such analyses can provide insight into the structural changes (direct or indirect) wrought by a particular gene's modification, these assays may not always throw light on the biological role of particular cell wall polymers, structural proteins and enzyme activities. Ultimately, we need to know how structural modifications of the cell wall are translated into functionally significant differences in cell wall properties and behavior. As mentioned above, the polar tip growth exhibited by protonemal cells represents a useful and highly sensitive system for estimating the effects of gene modification on cell wall growth and integrity. Even small differences in the rate of growth of extending protonemal cells can become apparent when integrated over time, and this can be useful for assaying genes whose deletion or mutation has only a small quantitative effect on cell wall biogenesis or extension. One problem encountered in studying cell wall mutants in higher plants is the degree of functional redundancy in the cell wall, such that removal of one component is compensated by the induction of another. Extreme examples include Arabidopsis mutants in cellulose biosynthesis that express high amounts of lignin as well as mutants that lack xyloglucans (Cavalier et al. 2008). This reflects the fact that mature plant cell walls contain a number of cross-linked polymers, each of which is capable of restraining the protoplasts and providing wall integrity. It is possible to explore (and certainly worth exploring) whether the immature cell wall present in the growing protonemal tip is be more highly sensitive to perturbations in individual cell wall components. Our own experience as well as that of other groups has been that the tip cell as well as tip cell growth is particularly sensitive to chemically and genetically induced changes in cell wall architecture (H. Saidasan, unpublished observations), (Lee et al. 2005b; Perroud and Quatrano 2008). *Physcomitrella* also allows the effects of cell wall

mutations to be assayed in the context of multicellular tissues, such as leaves and stems, and this allows aspects of the cell wall associated with development and with cell–cell signaling to be investigated.

Assays that are more directly related to the need of biofuel feedstocks include the use of digestive enzymes and chemical treatments to remove particular cell wall components or hydrolyze specific linkages and the use of pathogens and saprophytes to probe cell wall structure. Digestive enzymes and chemical treatments allow the physical consequences of cell modification to be queried and provide a direct way to screen mutants for modifications that (for example) may alter lytic enzyme access to cellulose and hemicellulose. One problem with the use of single lytic enzyme activities is that they may not be sufficient for digestion of the cell wall, which may require the concerted action of different digestive enzymes or chemical treatments (as is indeed required in commercial procedures for the digestion of plant cell walls). One way to approach this is to use pathogens and saprophytes (such as *Pantoea*, *Fusarium* or *Trichoderma* spp.) that produce a suite of lytic enzymes that can successfully digest different components of the plant cell wall. A related approach to uncovering mutant phenotypes is to combine genetic mutation in individual cell wall components with the use of chemical inhibitors that prevent or reduce the production of other cell wall components. In this way, the effects of single mutations that would otherwise be obscured by compensatory mechanisms can now be revealed.

7 Prospects and Outlook

The application of genetics, genomics, and bioinformatics together with more sensitive and efficient methods for determining the structure and architecture of the plant cell wall and its components have radically altered the research landscape in plant cell wall biology (for some recent elegant examples see (Paredez et al. 2008; Wyatt et al. 2008; Anderson et al. 2009)). Genomic methods are providing information at a rate that is challenging our ability to use it. In particular, these methods are providing a large number of leads in the form of genes associated with specific cell wall-related and other important traits. The pressing need is for efficient functional methods that can test the contribution of lead genes to specific traits. *Physcomitrella* provides a unique opportunity to characterize gene function through the creation of knockouts. It also provides an opportunity to more subtly mutate genes in situ, a feature that may be of some use for tailoring cell wall biosynthetic components with altered specificities or patterns of expression or localization. *Physcomitrella* is becoming more widely used as a system for examining gene function, and we can expect that it will continue to make important contributions to our understanding of basic processes in plant biology, including cell wall biogenesis. If its genetic potential is to be fully exploited, then it is essential that reverse genetic studies are accompanied by a more detailed structural characterization of the cell wall, as this would provide a biochemical and structural basis for interpreting phenotypes associated with specific gene knockouts. Accompanying this is a need for more widespread transcriptional studies so that

individual gene expression patterns are known. This can help select genes for mutation and also assist in interpreting the results of such studies. The recently developed methods for creating temperature-sensitive mutations in *Physcomitrella* should prove especially useful (Vidali et al. 2009). Because of its potential for genetic manipulation, *Physcomitrella* also offers the opportunity to construct new types of plant cell wall from the ground up. Such an approach has been successful for engineering and humanizing the *Physcomitrella* machinery of protein glycosylation (Weise et al. 2007) and it is reasonable, as we enter the era of synthetic biology, to begin to think about approaches for designing cell walls with features that are tailored for postharvest processing to biofuels. Thus, *Physcomitrella*, which lacks lignin and xylem, may represent a useful foundational system for introducing novel biosynthetic capabilities that confer the ability to synthesize this complex and important cell wall polymer. Finally, while *Physcomitrella* is likely to develop as a model system for functional studies of the cell wall, it is important that we simultaneously develop more efficient methods for transforming and modifying the genomes of species that can serve as biofuel feedstocks so that information obtained from model systems such as *Physcomitrella* and Arabidopsis can be applied to field crops.

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