

Chapter 2

Sources for Lignocellulosic Raw Materials for the Production of Ethanol

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Abstract Production of ethanol from non-crop plant cell walls represents a sustainable solution for biofuel production due to the abundance of these renewable resources on our planet. The resources for cellulosic raw material can be either dedicated bioenergy feedstocks such as fast growing trees and energy grasses, or those based on by-products and waste materials such as crop residues and municipal solid waste. The processing of lignocellulosic into biofuel still requires expensive and harsh pretreatments, some of which are not acceptable from environmental point of view. This is due to the high level crystallinity of the cellulose and cross linking of the carbohydrates with the lignin that form a barrier preventing efficient and economic biomass enzymatic digestion. The advances in plant genetic engineering enable genetic modifications of the plant cell wall structure and function and may provide solutions that will help to overcome the difficulty in utilizing energy crops and trees. Despite the current technological difficulties related to processing of the complex cell wall polymers into fermentable sugars, the demand for renewable liquid fuel motivates the search for practical solutions and development of innovative technologies.

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2.1 Introduction

Most ethanol produced to date as biofuel is generated from edible crops by fermentation of sugars from sugarcane (*Saccharum officinarum*) or conversion of starch from corn (*Zea mays*). However, this “first generation” approach led to the “*food versus fuel*” conflict and dilemma leading to search for alternative biomass sources for the “next generation biofuels” mostly based on cellulose (Kullander 2010; Valentine et al. 2012). Plant cell walls are the most abundant renewable resource on our planet with $150\text{--}170 \times 10^9$ tons produced annually (Pauly and Keegstra 2008). Thus, the production of ethanol from the cell walls of non-crop plants or non-edible parts of plants is considered a sustainable solution for biofuel production. This is despite the current difficulties related to the costs, high energy inputs, and harsh conditions required to process the complex cell wall polymers into fermentable sugars. The complex composition of lignocellulosic materials is a key factor affecting the efficiency of bioethanol production during the conversion processes (Jordan et al. 2012; Himmel et al. 2007; Dixon 2013). The major components of plant cell walls are cellulose, hemicellulose, and lignin that comprise around 90 % of its dry biomass (Gibson 2012; Harris and Stone 2008; Pauly and Keegstra 2008). The principal cell wall polysaccharide is cellulose, composed of hydrogen bonded chains of β -1,4-linked glucose. Cellulose is coated with hemicellulose. The most abundant type of hemicellulose is xylan, a polymer of β -1,4-linked xylose which may have branches containing other sugars such as arabinose or glucuronic acid, depending on the plant species. The saccharification of cellulose and hemicellulose releases glucose and xylose that can in turn be fermented to ethanol. Lignin, a complex polymer of hydroxylated and methoxylated phenylpropanoids, cross-links plant secondary cell walls to provide mechanical strength and hydrophobicity and it contributes to defense against pathogens. The percentage of lignin content in cell wall varies between plants and is a crucial parameter affecting the decomposition efficiency of the polysaccharides.

Among the various issues hampering competitive sustainable utilization of lignocellulosic materials for commercial fuel production, the choice of biomass feedstock is of major importance, and it is discussed in this chapter. Preferentially, biomass feedstock needs to be of high cellulose content and a non-crop species, such as fast growing trees or grasses that can grow on marginal soils. Such species would not compete for land use with food crops and could be grown at relatively dry zones. To reduce the environmental and economical impacts of their utilization, efforts should be made to use low quality water such as treated waste water, if

irrigation is required. These energy crops cell wall components can be modified via genetic engineering in order to facilitate hydrolyzing enzymes accessibility and reduce cost of pretreatments. Other sources of biomass are by-products, such as agricultural wastes or crop residues, agro-industrial by-products, and municipal solid waste (Limayem and Ricke 2012; Sanchez and Cardona 2008).

In addition to plant species and cell wall chemical composition, other requests for the choice of feedstock include: minimal energy input required for processing the biomass to ethanol; eco-friendly pretreatment of the lignocellulosic raw material; minimum production of yeasts inhibitors such as furfural and phenolic compounds by pretreatment; low price of production and processing of lignocellulose to fermentable sugars; stable supply of feedstock to ensure a continuous operation of the factory all-year round; optimized supply chain management from harvest to collection, storage, and transport on a local base. In addition, geography may dictate specific strategy for specific regions, i.e., different plants may be economically grown in a tropical area in comparison to temperate ones.

Faraco and Hadar (2011) focused on the potential of bioethanol fuel production from lignocellulosic residues in the Mediterranean Basin. Residues from cereal crops, olive trees, and tomato and grape processing are abundant lignocellulosic wastes in France, Italy, Spain, Turkey, and Egypt, where their use as raw materials for ethanol production could give rise to a potential production capacity of 13 Mtoe of ethanol. Due to the lack of sufficient amounts of agricultural residues in all the other Mediterranean countries, use of the cellulosic content of municipal solid waste as feedstock is also an option. A maximum potential production capacity of 30 Mtoe of ethanol could be achieved from 50 % of the 180 million tons of waste currently produced annually in the Mediterranean Basin (Faraco and Hadar 2011).

Another investigation showing the possibility to identify unique biomass sources on a local basis was conducted in North-East India (Sasmal et al. 2012). This study focuses on characterization of three plant species: nut husk (*Areca catheu*), moj (*Albizia lucida*), and bonbogori (*Ziziphus rugosa*), available in the analyzed region. Physical and chemical analysis of these lignocellulosic biomass samples showed that they can serve as potential sources for biofuel production.

In another study, Puri et al. (2012) analyzed the prospects, challenges, and feedstock for biofuel production in Australia, where the largest renewable resources for biofuel production revealed to be forest plantations, based on Eucalyptus trees, agricultural residues, and organic by-products, mostly bagasse.

Another important point of local nature should be the alternative use of the feedstock, either for energy production by other technologies or for different purposes such as animal feed or soil amendment. For example, wheat straw is considered a nuisance and inexpensive bioethanol feedstock in Europe and North America but it can be difficult to obtain during droughts in the Middle East.

Cellulosic raw materials including dedicated bioenergy feedstocks (trees and energy grasses) and those based on by-products and waste material (plant residues and municipal solid waste) are described in this chapter.

2.2 Annual and Perennial Dry Energy Grasses

Forages and particularly cultivated grasses can be considered prominent candidates for cellulosic biomass production and among the various forms of biomass available for ethanol production, they seem to be very promising as future biomass feedstock. Their cultivation and processing are the focus of widespread research. The major herbaceous energy crops that have been selected for bioethanol production are switchgrass (*Panicum virgatum*), miscanthus (*Miscanthus spp.* Anderss.), canary grass (*Phalaris arundinacea*), giant reed (*Arundo donax* L.), alfalfa (*Medicago sativa* L.), and Napier grass (*Pennisetum purpureum*). Besides avoiding “food versus fuel” conflict, they are considered to have energetic, economic, and environmental advantages over food crops for ethanol production (Hill et al. 2006; Chandel and Singh 2011). After a perennial grass is established, the major expenses are for nitrogen fertilizers and harvest. These plants can grow in marginal and erosive soils and respond to nitrogen fertilization with remarkable increase in biomass yield (Muir et al. 2001).

Switchgrass is native to North America, tall grass prairies are known for their rapid growth during the warm months to heights of two meters. Commercial switchgrass cultivation was studied mostly in the US and Canada. This plant can be grown in many environments, including swamplands, plains, and streams, and along the shores and interstate highways. It is self-seeding and resistant to many diseases and pests, and can produce high yields with low applications of fertilizer and other chemicals. It is also tolerant of poor soils, flooding, and drought; furthermore, it improves soil quality and prevents erosion due to its type of root system (Parrish and Fike 2009).

Miscanthus is another viable feedstock for cellulosic ethanol production. This species of grass is native of Asia and can grow up to 3.5 meters tall with little water or fertilizer inputs. It is similar to switch grass with respect to cold and drought tolerance and water use efficiency (Ng et al. 2010). *Miscanthus* is commercially grown in the European Union as a combustible energy source (Brosse et al. 2012). The cellulose and lignin levels vary between the different crops of *Miscanthus* with the highest cellulose content in range of 40 % (Chandel and Singh 2011), suggesting its highest potential for ethanol yield.

Napier grass is tropical grass native to the African grasslands. It requires very little supplement of nutrients for growth. It can be harvested 3–4 months after planting and then at intervals of 6–8 weeks for up to 5 years with a annual dry biomass yield per hectare of 40 tons (Woodard and Prine 1993). Its fibers can be used for obtainment of polymer derivatives and composites in addition to bioethanol (Reddy et al. 2012). It was suggested to use Napier grass, in addition to other grasses, in several tropical countries including countries like Thailand, Philippines, Kenya, and Brazil (Wongwatanapaiboon et al. 2012). In addition to the high biomass produced, it has been shown that hybrid giant Napier grass is an effective salt tolerant plant that can grow well in saline soil and reduce the saline soil pH (Ma et al. 2012).

The bioethanol industry needs a continuous and reliable supply of biomass that can be produced at a low cost and with minimal use of water, fertilizer, and arable land. Byrt et al. (2011) reviewed several studies focusing on a number of key existing and potential energy crops which are C_4 plants, and compared the photosynthesis rate, the composition of the plant cell wall as well as use of water, fertilizer, sugar yields, biomass yields, and the calculated ethanol production yield. They showed, based on calculations, that ethanol yields from C_4 sugar crops, such as sugarcane, sugar beet, and sweet sorghum may be ranging from 4000 to 8000 L/Ha, and exceed the ethanol yields from starch from grain crops. However, data directly comparing the performance of potential biofuel feedstock crops under different climate and field conditions is lacking. As pointed out by Byrt et al. (2011), these data should be collected regionally, to be able to assess which crop may give the highest bioethanol yield under different environments. They proposed that plant scientists have not yet even started to explore the rich genetic resources available for improving C_4 grasses and breeding of biofuel crops such as miscanthus and switchgrass, sorghum and sugarcane. C_4 biofuel feedstock improvement is only in its infancy considering the efforts invested in agricultural selection and breeding of corn over thousands of years (Byrt et al. 2011).

2.3 Forest Woody Feedstock

Fast growing short rotation forest trees can play an important role as feedstock for bioenergy production (Seguim 2011). However, forests are unevenly distributed. Forests play important environmental role in preservation of marginal land and reducing CO_2 levels in the atmosphere. Forest woody feedstock account for approximately 370 million tons per year of lignocellulosic biomass in the US (Perlack et al. 2005), other countries rich in forests are for example, Canada, the Russian Federation, Brazil, and China. Together, these countries account for more than half of the total forest area worldwide. Sources of woody materials include residues left in natural forest, forestry wastes, such as sawdust from sawmills, wood chips and branches from dead trees, and cultivated short rotation energy forest plantations utilizing several fast growing tree species.

There are two types of woody materials, softwoods, or hardwoods. Softwoods originate from conifers and gymnosperm trees (Sanchez and Cardona 2008). Unlike hardwoods, softwoods possess lower densities and grow faster. These trees comprise of evergreen species such as pine, cedar, spruce, cypress, fir, hemlock, and redwood. Hardwoods are mainly found in the Northern hemisphere and include trees such as poplar, willow, oak, cottonwood, and aspen. In the US, hardwood species account for over 40 % of the trees (Perlack et al. 2005). The genus *Populus* (cottonwood) which includes 35 species is the most abundant fast growing species suitable for bioethanol production. An advantage of woody biomass over agricultural plants is the flexibility in harvesting times as they do not depend on seasonality. Trees also contain less ash compared to crops and are of

higher density, due to the thick secondary wall, which makes their transportation more economical.

Nieminen et al. (2012) reviewed the different options for optimizing wood development in bioenergy trees. They concluded that tree breeding has been, thus far, very challenging due to the trees' long generation time. However, new breeding possibilities are emerging through the development of high-throughput technologies in molecular genetics. They describe traits, including stem morphology and xylem cell dimensions that could be modified to enhance wood production as well as hormonal and molecular regulation of wood development (Nieminen et al. 2012).

2.4 Municipal Solid Waste

In the European Union countries, over 250×10^6 tons of Municipal Solid Waste (MSW) are produced each year, with an annual growth of 3 %. In 1990, each individual in the world produced an average of 250 kg of MSW generating in total 1.3×10^9 tons of MSW (Beede and Bloom 1995). Ten years later, this amount almost doubled leveling at 2.3×10^9 tons (Al-Salem et al. 2009). MSW treatment and recycling in general have both economical and environmental implications that should be considered also for ethanol production (Chester and Martin 2009; Kalogo et al. 2007; Stichnothe and Azapagic 2009). In many countries, the MSW is separated into several fractions, thus the biodegradable organic components of MSW consisting of paper and cardboard, kitchen waste and garden waste, could be converted into biofuel (Li et al. 2007), although these are not an ideal feedstock, due to the diversity in the MSW components and less than perfect source separation and existence of contaminations. However, it may be useful in regions where more suitable raw materials are lacking or scarce. For example, with the advances in cellulosic ethanol technologies, the Mediterranean could use the cellulosic content of MSW as a transportation fuel feedstock and simultaneously reduce externalities associated with land filling. It was calculated by Faraco and Hadar (2011) that if assuming between 60 and 90 % practical yields for ethanol production, the Mediterranean could produce between 17 and 25 billion liters per year of ethanol from 50 % of the 180 million tons of waste currently produced annually. The organic fraction of MSW contains lignocellulose in the form of waste paper products and food residues which could be an adequate raw material for ethanol production (Schmitt et al. 2012). For example, Li et al. (2012) achieved 53 % conversion of the cellulose and hemi-cellulose by enzymatic hydrolysis in buffer solution containing 6 wt % lignocellulosic MSW concentrate incubated at 40 °C for 12 h. An important parameter for successful hydrolysis was the particle size ranging 150–300 μm . Li et al. (2012) suggested that 1 ton of the fiber originated from MSW can produce 154 L of bioethanol.

In addition to bioethanol, energy can be produced from MSW via incineration or anaerobic digestion and production of CH_4 -enriched biogas. Another useful product produced from the organic fraction on MSW is compost applied as soil conditioner.

2.5 Agricultural Residues

Agricultural crop residues include field residues and processing residues. Agricultural wastes are renewable and abundant resources. Field residues represent materials left in an agricultural field after harvesting the crop, and they include straw and stalks, leaves, and seed pods. Processing residues, such as husks, seeds, bagasse and roots, are those materials left after the processing of the crop into a usable resource. Harvesting of cereals, vegetables, and fruits generates huge amounts of crop residues.

Rice straw, wheat straw, corn stover, and sugarcane bagasse are the major agricultural wastes in terms of quantity of biomass availability (Kim and Dale 2004). Kim and Dale (2004) estimated that there are 73.9×10^6 ton of dry wasted crops in the world that could potentially produce $49.1 \text{ GL year}^{-1}$ of bioethanol. About 1.5×10^9 ton year^{-1} of dry lignocellulosic biomass from seven crops is also available for conversion to bioethanol. The total potential bioethanol production from crop residues and wasted crops is 491 GL year^{-1} (Kim and Dale 2004).

Sugarcane is among the principal agricultural crops cultivated in tropical countries. Bagasse is the residue obtained from the sugarcane after it is crushed to obtain the juice used for sugar and ethanol production, thus it has an advantage over other crop residues as it is a by-product, already collected to the ethanol refinery and does not require special and costly collection and transportation. The annual world production of sugarcane is 1.6 billion tons, and it generates 279 million tons of biomass residues (bagasse and leaves). For the past three decades, bagasse and leaves have been explored for use in lignocellulosic bioconversion (Chandel et al. 2012). Theoretically, one ton of bagasse could yield up to 300 L of ethanol. In addition to ethanol, sugarcane bagasse has been successfully converted into many value-added products such as xylitol, organic acids, and industrial enzymes (Chandel et al. 2012).

In addition to the crops cultivated globally, some crops have local importance. Nevertheless, they can be very significant for the region economy and a substantial source of biomass for biofuel. An example is represented by olive groves (Faraco and Hadar 2011). Cultivation of olives in the Mediterranean results in huge amounts of lignocellulosic residues (olive tree pruning residues and olive mill solid waste).

Olive oil production represents one of the most important economic agro-food sectors in the Mediterranean Basin. Southern Europe (Spain, Italy, and Greece) is the world's largest producer of olive oil, accounting for 79 % of world olive oil

production in 2005, when they produced around 2 million tons of olive oil (UNCTAD 2007). The olive oil industry generates two downstream by-products, olive mill solid waste, and olive mill wastewater which pose environment hazard and pollution. Crude olive mill solid waste, the leftover solids following the pressing of olives, contains a mixture of skin, pulp, and seeds. It comprises approximately 35 % of the olives starting weight. The newer two-stage extraction technology significantly reduces the amount of wastewater produced, but generates a new type of waste, a solid residue called olive pulp (OP) resulting in approximately 8 million tons of OP generated per year in Southern Europe (UNCTAD 2007). At present, OP is either discarded to the environment or combusted with low economic value.

The use of all of the residues generated from both harvesting and processing of olives in the Mediterranean as raw materials for ethanol production could be of importance for the countries involved. Several authors analyzed the technological feasibility and potential of converting wastes from the olive industry to ethanol. Based on recent literature, Faraco and Hadar (2011) analyzed the feasibility and potential of olive trees pruning as well as olive mill waste as a source for fermentable sugars.

A major concern in the utilization of crop residues is the residue collection methodology and efficiency. According to Perlack et al. (2005), most operations are needed to pick up the residues left on the ground after the crops have been harvested. Collection of residues from these crops involves multiple passes of equipment over fields and results in removal of only 40 % of the biomass. Perlack et al. (2005) envisaged future residue collection technology with the potential of collecting up to 75 % of the residues. The equipment to be developed is expected to be single-pass system that would reduce costs by collecting the grain and residue together and reduce soil compaction. As with other raw materials, when discussing the use of crop residues as raw material for biofuel, their alternative uses should be considered. Of special importance is their use as soil conditioner and for increasing the levels of soil organic matter, with important effects on soil structure, preventing erosion, the supply of nutrients, acidification, and water-holding capacity of soils, all affecting soil fertility and health (Lal 2005; Recosky and Forcella 1998; Tarkalson et al. 2006; Wilhelm et al. 2004). These effects can be maintained harvesting only fraction of the residues or by returning the left-over from the processed biomass to the field. These residues are rich in lignin and also contain unreacted cellulose and hemicellulose (Mosier et al. 2005).

Besides ethanol, biodiesel, produced from plants, such as *Jatropha curcas*, or algae rich in lipids is considered an important future biofuel. Although biodiesel is not the focus of this paper, it should be mentioned that once the lipids have been extracted, the leftover solids are composed of mostly carbohydrates. These carbohydrates are potential substrates that could be fermented to produce ethanol. In such a case, several biofuels are produced from one biomass source (Jones and Mayfieldt 2012).

In conclusion, the differences and variation between the many crop residues, some of which available on local basis only and during different seasons, require the development of flexible conversion processes.

2.6 Cell Wall Engineering

Although the availability of renewable cellulosic feedstock is almost unlimited around the globe, the processing of lignocellulose into fermentable sugars and biofuel still requires expensive and harsh pretreatments, due to the high level crystallinity of the cellulose and cross linking of the carbohydrates with the lignin that form a barrier preventing efficient and economic biomass enzymatic digestion. Some pretreatments are not acceptable from environmental point of view. The advances in plant genetic engineering enable genetic modification of plants and this is widely used in commodity crops such as corn, soybean, and cotton. Safe and environmentally accepted genetic engineering technology can be used to modify cell wall structure and function and may provide a solution that will help to overcome the difficulty in utilization energy crops and trees. Limited number of environmental studies conducted so far, failed to indicate any strong effects of lignin-modified transgenic trees on the ecosystem (Pilate et al. 2012). Nevertheless, Nonic et al. (2012) reviewing the possible use of genetically modified trees in EU countries concluded that it is important to develop recommendations for the use of genetically engineered trees for forestry and plantations, taking into account socio-economic analyses as well as acceptance by the public. Indeed, as the cell wall structure and composition is providing the plant strength, defense mechanism against pathogens, and protection against other biotic and abiotic stress, the genetic modification of plant cell walls could unexpectedly lead to alteration of plant growth and development and result in harmful effects such as poor plant fitness (Jung et al. 2012b). Nevertheless, large efforts and investments are made in recent years toward this direction. The various possibilities for improvement of plant performance as feedstock for ethanol production were reviewed in recent years (Abramson et al. 2010; Xie and Peng 2011; Wang and Brummer 2012; Mizrachi et al. 2012; Jung et al. 2012b; Cook and Devoto 2011). In addition to modifying genes directly related to cell wall synthesis, another approach to increase the suitability of a crop as a feedstock is to increase biomass yield or increase plant productivity in general by affecting photosynthesis rate by over-expressing genes like phosphoenolpyruvate carboxylase, fructose-1, 6-bisphosphatase and sedoheptulose-1, 7-bisphosphatase (Lefebvre et al. 2005). Enhancement of trees growth and performance via genetic engineering for biomass production was reviewed by Harfouche et al. (2011).

The genes that can be modified include structural genes as well as transcription factors that function as positive or negative regulators of lignin or cellulose synthesis (Wang and Dixon 2012).

2.7 Targeting Lignin Biosynthesis

A prerequisite for lignin structure and function modification is deep understanding of its biosynthetic pathway and the ability to predict the outcome of alternation of any of the many genes involved in the process. Vanholme et al. (2012a) reviewed the current knowledge on lignin and its precursors' biosynthesis and discussed the characteristics of alternative lignin monomers and criteria to meet for the purpose of increased susceptibility of the biomass to the depolymerization. They described in detail several types of compounds that may be used as alternative monomers for lignin biosynthesis: monomers that directly produce a readily cleavable functionality in the polymer; hydrophilic monomers; monomer conjugates linked via a readily cleavable functionality; monomers that minimize lignin–polysaccharide cross linking and monomers that give rise to shorter lignin polymers (Vanholme et al. 2012a).

Eudes et al. (2012) described a strategy developed in *Arabidopsis* for the overproduction of rare lignin monomers to reduce lignin polymerization degree via incorporation of side-chain-truncated lignin monomers. In this work, the expression of the bacterial hydroxycinnamoyl-CoA hydratase-lyase (HCHL) in *Arabidopsis* was restricted to the lignifying tissues using a secondary cell wall-specific promoter, thus avoiding adverse phenotypes observed in previous studies. HCHL cleaves the propanoid side-chain of hydroxycinnamoyl-CoA lignin precursors to produce the corresponding hydroxybenzaldehydes so that plant stems expressing HCHL accumulate in their cell wall higher amounts of hydroxybenzaldehyde and hydroxybenzoate derivatives. Engineered plants did not show reduction in total lignin, sugar content, or biomass yield compared with wild-type plants. However, cell wall analyses revealed an increased amount of unusual C₆C₁ lignin monomers and lignin with a reduced molecular weight. These plants showed an increase in saccharification of pretreated stem cell walls (Eudes et al. 2012).

Jung et al. (2012a) achieved a reduction of the recalcitrance of sugarcane biomass by reducing lignin content via RNA interference suppression of lignin biosynthesis. Downregulation of the sugarcane caffeic acid *O*-methyltransferase (*COMT*) gene by 67–97 % reduced the lignin content by 3.9–13.7 %, respectively. The syringyl/guaiacyl ratio in the lignin was reduced from 1.47 in the wild type to values ranging between 1.27 and 0.79. The yields of fermentable glucose were 96 and 135 mg glucose g⁻¹ in the wild type and the best transgenic plant, respectively, without pretreatment. After dilute acid pretreatment, the fermentable glucose yield was increased to 190 and 288 mg glucose g⁻¹ in the wild type and the best transgenic plant, respectively. These observations demonstrate that a moderate reduction in lignin can reduce the recalcitrance of sugarcane biomass without compromising plant performance (Jung et al. 2012a).

The lignin content of a feedstock has been proposed as one of the key agronomic traits impacting biofuel production from lignocellulosic biomass. 4-Coumarate:coenzyme A ligase (4CL) is one of the key enzymes involved in the monolignol biosynthetic pathway. Xu et al. (2011) showed that silencing of 4CL in

switchgrass leads to reduced lignin content and improved fermentable sugar yields. RNA interference of *Pv4CL1* reduced extractable 4CL activity by 80 %, leading to a reduction in lignin content with decreased guaiacyl unit composition. Altered lignification patterns in the transgenic plants did not compromise biomass yields. Dilute acid pretreatment of the low lignin transgenic biomass resulted in significant increase of 57 % in cellulose hydrolysis efficiency (Xu et al. 2011).

As was shown in the aforementioned examples, engineering of a specific gene in the lignin biosynthetic pathway can lead to positive results. However, lignin engineering may also results in profound metabolic consequences in the plant. Vanholme et al. (2010, 2012b) used a systems biology approach to study the response of the plant to lignin disruption. They studied 20 *Arabidopsis thaliana* mutants, each mutated in a single gene of the lignin biosynthetic pathway and analyzed them using transcriptomic and metabolomic approaches. By combining metabolomic and transcriptomic data in a correlation network, system-wide consequences of the perturbations were revealed and genes with a putative role in phenolic metabolism were identified. Together, these data provide insight into biosynthesis of lignin and the metabolic network it is embedded in, and provide a system view of the plant's response to pathway perturbations (Vanholme et al. 2012b). This system biology study was performed using the model plant *Arabidopsis*, rather than on a specific energy crop. However, it is reasonable to assume that it is feasible to translate cell wall research from *Arabidopsis* to commercial crops. In addition, the existing *Arabidopsis* mutant collections and natural accessions are the best available genetic bases to reveal, through systems biology, how mutations in cell wall recalcitrance genes affect biosynthesis in other metabolic and developmental processes—information that will be crucial for the rational design of bio-energy crops (Vanholme et al. 2010). Other examples are discussed by Pilate et al. (2012) with emphasis on the evaluation of performance of transgenic trees in field trial for assessing the effects of lignin modification on wood properties and trees physiology and performance. They suggest a threshold of about 20 % reduction in lignin content to avoid negative effects such as winter mortality of the transgenic trees. Evaluation under field conditions is of great importance for the identification of new directions to improve wood properties for applications such as pulp and paper manufacturing and ethanol production (Pilate et al. 2012).

2.8 Targeting Cellulose Structure and Function

Cellulose is naturally resistant to enzymatic hydrolysis. The chains of covalently linked glucose molecules form microfibrils which have a firmly condensed structure. Cellulose microfibrils are insoluble in water; therefore cellulolytic enzymes have a small surface area to act upon. Reducing the recalcitrance of cellulose to enzymatic hydrolysis into a fermentable form of sugar via decreasing

the crystallinity and increasing the accessibility into the glucan chain is the aim of many studies.

Overexpression of glycoside hydrolases was suggested as a mean to modulate the cell wall. The *Arabidopsis* endo-(1-4)- β -glucanase protein (Cel1) accumulates in young, expanding tissues, playing a key role in disruption of cell wall during cell elongation of rapidly growing tissues (Shani et al. 1997; Shani et al. 2004). Heterologous overexpression of *cel1* in poplar trees or of poplar endoglucanase (*PaPopCell*) in *Arabidopsis* resulted in longer internodes, increased cell elongation, and subsequent biomass accumulation (Park et al. 2003; Shani et al. 2004). Mechanical analysis, studying leaf blade extension at constant load and breakage at changing load, was conducted. An elongation *versus* load curve demonstrated higher elongation rates in transgenic *Arabidopsis* leaf blades when compared to wild type, it was speculated that the cell wall of these transgenic plants contained less cross-linked polymers (Park et al. 2003). Similar results were obtained upon expression of *Aspergillus niger* xyloglucanase in poplar trees. Both stem length and cellulose content increased (Park et al. 2004).

In conclusion, overexpression of endoglucanases can enhance plant growth, but may also result in undesirable effects; therefore attempts should be made to use tissue-specific promoters for targeting the overexpressed gene.

Cellulose binding modules (CBM) expression is another mean to enhance cell wall biosynthesis. Plant growth and biomass can be increased by bacterial CBMs transgenically expressed in the cell wall (Shoseyov et al. 2006). Shoseyov et al. (2006) suggested that this effect is the result of separation of the cellulose-biosynthesis polymerization and crystallization steps. Accelerated cell and plant growth have also been observed in transgenic tobacco, poplar, and potato plants expressing a cell wall-targeted *Clostridium cellulovorans* CBM (Levy et al. 2002; Safra-Dassa et al. 2006). The role of CBM expression in increased plant growth rates could have been potential in yield enhancement and can be applied to many biofuel feedstocks. This genetic modification can change the carbon partitioning between source and sink tissues by creation of stronger sinks in cellulose synthesizing cells, leading to enhanced growth, biomass, and yield (Abramson et al. 2010).

Harris et al. (2009) genetically modified the cellulose synthase of *Arabidopsis* and reduced the crystallinity of cellulose and improved its biochemical conversion to fermentable sugars. In the mutant studied, a 34 % lower biomass crystallization index and 151 % improvement in the efficiency of conversion from raw biomass to fermentable sugars was measured, relative to that of the wild type. They later showed that the cellulose microfibril crystallinity was reduced by mutating the C-terminal transmembrane region residues of cellulose synthase (Harris et al. 2012).

Manipulation of cellulose synthase can result in negative effects. Joshi et al. (2011) studied the possibility of overexpression of an aspen secondary wall-associated cellulose synthase gene in transgenic aspen (*Populus tremuloides* L.) but they unexpectedly observed silencing of the transgene as well as its endogenous counterparts. The main axis of the transgenic aspen tree stopped growing, and weak branches showed a weeping growth pattern. The transgenic stems containing

reduced amounts of crystalline cellulose produced typical collapsed or irregular xylem vessels that had altered secondary wall morphology. These results demonstrate the fundamental role of secondary wall cellulose within the secondary xylem in maintaining the strength and structural integrity required to establish the vertical growth of trees. In another study, Hoenicka et al. (2012) studied the effect of overexpression of the flowering promoting factor 1 gene (*FPF1*) from *Arabidopsis* on wood formation in hybrid poplar. They found a strong effect on wood formation but no effect on flowering time. Wood density was lower in the transgenic plants, despite the significantly reduced vessel frequency which was compensated by thinner fiber cell walls. Chemical screening of the wood by pyrolysis GC/MS showed that *FPF1* transgenic plants have higher fractions of cellulose and glucomannan products as well as lower lignin content.

As demonstrated above, cellulose and lignin biosynthetic pathways have been extensively studied. However, modifications of other wall matrix components during secondary growth have been the focus of only few studies, despite their importance as the third component of the secondary cell wall which is cross-linked to both lignin and cellulose (Cook and Devoto 2011; Park et al. 2004; Lee et al. 2009). For example, Bindschedler et al. (2007) studied the downregulation of UDP-glucuronate decarboxylase (the enzyme responsible for UDP-xylose synthesis) in transgenic tobacco. Several of the down-regulated antisense plants showed high glucose to xylose ratios in xylem walls due to less xylose-containing polymers. However, unexpectedly, this result did not lead to improvements in cellulose extractability. Goulao et al. (2011) observed differential expression of several genes involved in hemicellulose and pectin in xylem or phloem of stem regions undergoing secondary growth in *Eucalyptus globulus*. These results suggest that hemicellulose and pectin biochemistry in wood formation and architecture could be a target for gene manipulations. It seems that more attention should be given to the research in this direction.

2.9 Concluding Remarks

The growing demand for liquid fuel accelerates the research and applications in the areas of development and exploitation of cellulosic raw materials. It is estimated that as much as 1.3 billion dry tons of biomass could be produced annually in the US only, by 2030 (Perlack et al. 2005). If 90 % of the sugars derived from the biomass will be utilized for bioethanol production, it will be sufficient for approximately 130 billion gallons of cellulosic ethanol, equivalent on an energy basis to approximately 87 billion gallons of gasoline (Carroll and Somerville 2009). Beringer et al. (2011) calculated the global bioenergy potentials of biomass sources under environmental and agricultural constraints and concluded that they may provide between 130 and 270 EJ year⁻¹ in 2050, equivalent to 15–25 % of the World's future energy demand. The sources for cellulosic feedstocks are diverse and include dedicated bioenergy crops (tress and energy

grasses) and by-products and waste material (agricultural wastes, plant residues, and municipal solid waste). The use of agricultural wastes and by-products is of special importance, from environmental point of view, since it increases the efficiency of the utilization of the solar energy converted by crop plants without exploitation of additional natural resources such as land and water. Although the principles of utilization of plant biomass are ubiquitous, the choice of feedstock either plant species or waste material depends on local conditions and economy. These new potential markets for biofuels can provide new economic possibilities to rural and remote regions. However, a whole life-cycle assessment (LCA) is required to ensure that social and potential environmental problems in addition to financial performance are taken into account. Inconsistencies in the assumptions applied to biofuel LCA and lack of standardization may lead to variable and even conflicting estimates (Davis et al. 2009). Misleading estimates may impair the sustainability of cellulosic ethanol.

Despite the availability of the lignocellulosic biomass, the processing into fermentable sugars and to biofuel still requires expensive and harsh pretreatments, some of which are not acceptable from environmental point of view. This is the result of the high level crystallinity of the cellulose and cross linking of the carbohydrates with the lignin that form a barrier that prevents efficient enzymatic digestion. Development of improved analytical techniques and enquiring additional basic knowledge on the interrelationship among the major cell wall components, hemicellulose, lignin, pectin, and cellulose could be an important step toward improvement of energy crops (Jordan et al. 2012). The advances in plant genetic engineering enable genetic modification of plant cell wall structure and function and may provide a solution that will help to overcome the difficulty in utilization energy crops and trees. The major advances in crop plant genetic molecular biology may accelerate this trend, however, cell wall composition, structure, and function have not been the focus in the breeding and molecular studies of most food crops, where the focus is on yield quantity and quality and resistance to pests, thus much basic knowledge is missing in that area and can be a fertile topic for research (Carroll and Somerville 2009). However, this should be done with caution as designing plants for improved degradability may result in adverse effects on plant development and survival. The ultimate goal should be “matching optimized feedstock traits (phenotypes) with low-input processing technology” (Ellis 2012). Using genetic engineering technologies will require caution to avoid undesirable traits and should also address ecological concerns.

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