Tansley review

Sustainable liquid biofuels from biomass: the writing's on the walls

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Summary

Domination of the global biosphere by human beings is unprecedented in the history of the planet, and our impact is such that substantive changes in ecosystems, and the global environment as a whole, are now becoming apparent. Our activity drives the steady increase in global temperature observed in recent decades. The realization of the adverse effects of greenhouse gas emissions on the environment, together with declining petroleum reserves, has ensured that the quest for sustainable and environmentally benign sources of energy for our industrial economies and consumer societies has become urgent in recent years. Consequently, there is renewed interest in the production and use of fuels from plants. The 'first-generation' biofuels made from starch and sugar appear unsustainable because of the potential stress that their production places on food commodities. Second-generation biofuels, produced from cheap and abundant plant biomass, are seen as the most attractive solution to this problem, but a number of technical hurdles must be overcome before their potential is realized. This review will focus on the underpinning research necessary to enable the cost-effective production of liquid fuels from plant biomass, with a particular focus on aspects related to plant cell walls and their bioconversion.

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I. Historical context

The development of human societies is inextricably linked to plants. The energy fuelling terrestrial life processes is initially captured from sunlight by photosynthesis, fixed into carbon-based molecules, and gradually released again through the metabolism of these energy-rich molecules. This is an ecosystem-wide economy of energy transfer using bonds formed and broken between carbon, hydrogen and oxygen as vectors for energy transfer. This process forms the basis of the global carbon cycle and the energy transduced in it serves to fuel the living processes of the planet.

Human beings have optimized the use of this energy by cultivating close relationships with 'useful' plants (crops) and adapting much of the terrestrial environment to the dedicated production of these species, thus allowing a greater proportion of globally fixed solar energy to be used for our societies. Much of our early industrial development was fuelled by the energy stored in plant biomass, mostly released by combustion and transduced in increasingly sophisticated ways such as the use of steam engines. However, the industrial revolution gained full momentum through the exploitation of vast subterranean reserves of fixed carbon molecules stored in the form of fossil fuels such as coal and petroleum. These fossil reserves (the products of historic photosynthesis) were laid down over millions of years and, along with the calcium carbonate exoskeleta of marine organisms, have formed the long-term carbon sinks responsible for the gradual decline in CO_2 concentrations over the geological history of the planet.

The development of our industrialized societies has had two synergistically related effects: on one side the depletion of oil reserves, and on the other the release of the stored fossil CO_2 into the atmosphere. The link between greenhouse gas emissions (particularly CO_2) and global warming is now generally accepted by most authorities working in the area of climate studies, as well as most creditable politicians. In addition to the overall concern regarding the impact of human activity on the climate, global energy policy is also being shaped by the declining reserves of petroleum and the rapid increase in demand for this commodity as more nations become industrialized and affluent.

II. The case for liquid biofuels in the context of human energy consumption

The burning of fossil fuels for the generation of heat and energy represents the greatest source of anthropogenic carbon emissions, and the replacement of these fossil fuels with more carbon-neutral and renewable sources has become a key objective for policy makers in developed and developing economies alike. Human energy consumption can be broadly grouped under three headings: heat, grid electricity, and transportation fuels, and each of these is affected by a different set of considerations, which define the options available for improving sustainability and minimizing environmental impacts (Pehnt *et al.*, 2006). Although plant biomass can, and does, make an important contribution in all three areas, it is in the context of transportation fuels that plant and microbial sciences have the biggest opportunity to make an impact, and therefore this review will address only that particular area.

Almost all of the transportation systems operating in developed countries are driven by internal combustion engines which use volatile liquid fuels. Indeed, liquid transportation fuels account for approx. 30% of the carbon emissions in industrialized countries and, as such, they are a major target in the drive to cut carbon emissions and increase sustainability in fuel supplies. There is a general belief that in the long term there will be a switch to the use of fuel cells and the so-called hydrogen economy. However, such a switch is not likely in the medium term because of the high cost of developing the infrastructure (and its environmental impact), as well as the overall inertia in the industries that manufacture and sell the transportation systems themselves. In addition, current methods for hydrogen production are inefficient, and some have a worse carbon footprint than burning petroleum-derived fuels (http://www.transportation.anl.gov/pdfs/TA/165.pdf). Because of this it is essential, in the short and medium term, that we find more sustainable means of generating liquid transportation fuels.

III. Liquid biofuels

Currently, 98% of transportation fuels are derived from petroleum, with the subsequent negative attributes in terms of fuel security and carbon emissions. Consequently, there has been renewed interest in the production and use of liquid biofuels such as biodiesel, which is produced from plant or animal oils, and bioethanol, which is produced by fermentation. Biofuels are renewable and can be produced from agricultural products with the potential to lessen the dependence on fossil fuels. Biofuels also have, in principle, lower carbon emissions because they are produced within the short-term carbon cycle, and their combustion only returns as much CO_2 to the atmosphere as plant growth has taken out. Hence, unlike the burning of fossil fuels, the combustion of biofuels has the potential to be carbon neutral.

1. First-generation biofuels and the problem of food security

The dramatic rise in oil prices seen in the last decade has also enabled liquid biofuels to become cost-competitive with petroleum-based transportation fuels, and this has led to a surge in research and production around the world. At present, bioethanol and biodiesel are both produced from commodities that are also used for food. These are referred to as first-generation biofuels. In the main, biodiesel is made from the same oil crops used in the food industry, and bioethanol

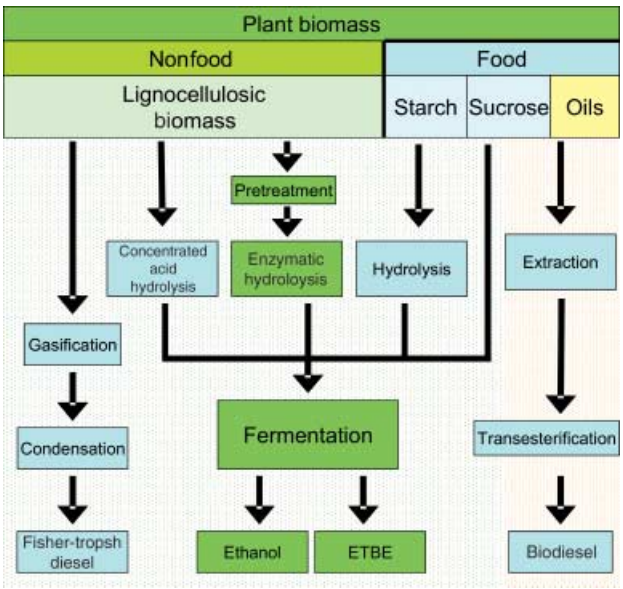

Fig. 1 Liquid biofuel production from biomass. ETBE, ethyl tertiary butyl ether.

is produced by the fermentation of sugars from sugar cane (*Saccharum officinarum*) or sugar beet (*Beta vulgaris*), or derived from the hydrolysis of starch (Fig. 1). More than 200 yr ago Malthus established the concepts of tensions between food supply and population, and, despite the great gains made in the 'green revolution' and the more recent impact of agricultural biotechnology, it is still possible that it may become increasingly difficult to feed the rapidly growing human population. It is against this background that the use of food commodities for biofuel production has generated concern. Debate about the 'food/fuel dilemma' has gained momentum as the recent increase in bioethanol production, particularly in the USA, has already led to marked increases in food prices (Odling-Smee, 2007). At present, opinion is divided about the long-term consequences of the use of agricultural products to replace fossil fuels. A cautious view of the benefit of biofuels is supported by a comprehensive assessment which suggests that the human species already appropriates a remarkably high proportion of the Earth's primary productivity (Haberl *et al.*, 2007). Conversely, a recent study evaluating a scenario in 2050 where food and biofuel production compete suggests that highly efficient agricultural systems will reduce the area of land used at present for food production by as much as 72% (Smeets *et al.*, 2007). The risks and opportunities offered by future changes in the agricultural landscape are currently being evaluated by the United Nations' Bioenergy and Food Security Project (http://www.fao.org/nr/ben/befs).

In addition to the food/fuel dilemma, sugar and starch crops require very substantial inputs of fertilizers and pesticides, and life-cycle analyses indicate that the production of bioethanol from corn has a net CO_2 emission rather than being carbon neutral (Hill *et al.*, 2006; Runge & Senauer, 2007). In contrast, the production of cellulosic bioethanol from plant biomass has the potential to be carbon negative and to avoid the conflict between food and fuel production (Tilman *et al.*, 2006).

2. Second-generation biofuels

Second-generation biofuels are produced from biomass in a more sustainable fashion, which is truly carbon neutral or even carbon negative in terms of its impact on CO_2 concentrations. In the context of biofuel production, the term 'plant biomass' refers largely to lignocellulosic material as this makes up the majority of the cheap and abundant nonfood materials available from plants. At present, the production of such fuels is not cost-effective because there are a number of technical barriers that need to be overcome before their potential can be realized. Plant biomass represents one of the most abundant and underutilized biological resources on the planet, and is seen as a promising source of material for fuels and raw materials (US DOE, 2006). At its most basic, plant biomass can simply be burned in order to produce heat and electricity. However, there is great potential in the use of plant biomass to produce liquid biofuels. Plant biomass is comprised mostly of plant cell walls, of which typically 75% of this material is composed of polysaccharides. These polysaccharides represent a valuable pool of potential sugars, and even in traditional food crops such as wheat (*Triticum aestivum*) there is as much sugar tied up in the stems as there is in the starch of the grains. To date, the potential of many crop residues, such as straw and wood shavings, to provide sugar feedstocks for biofuel production has not been realized. However, biofuel production from agricultural by-products could only satisfy a proportion of the increasing demand for liquid fuels. This has generated great interest in making use of dedicated biomass crops as feedstock for biofuel production.

Dedicated biomass crops are plants that are able to produce very substantial yields of biomass in a short period of time, when grown on marginal lands or with minimal input of fertilizers and pesticides. A range of different biomass crops are being considered for widespread growth for biofuel applications. These include perennial C_4 grasses such as *Miscanthus* × *giganteus* and switchgrass (*Panicum virgatum*), as well as woody species such as poplar and willow. The choice of crop for biomass production depends largely on the growth conditions of a particular region. There are many important areas where research is needed in relation to dedicated biomass crops, including optimization of varieties and the agronomics of production, as well as the environmental impacts of growing such crops.

IV. Converting plant biomass into liquid fuels

There are two main routes available for producing liquid biofuels from biomass; one involves thermochemical processing

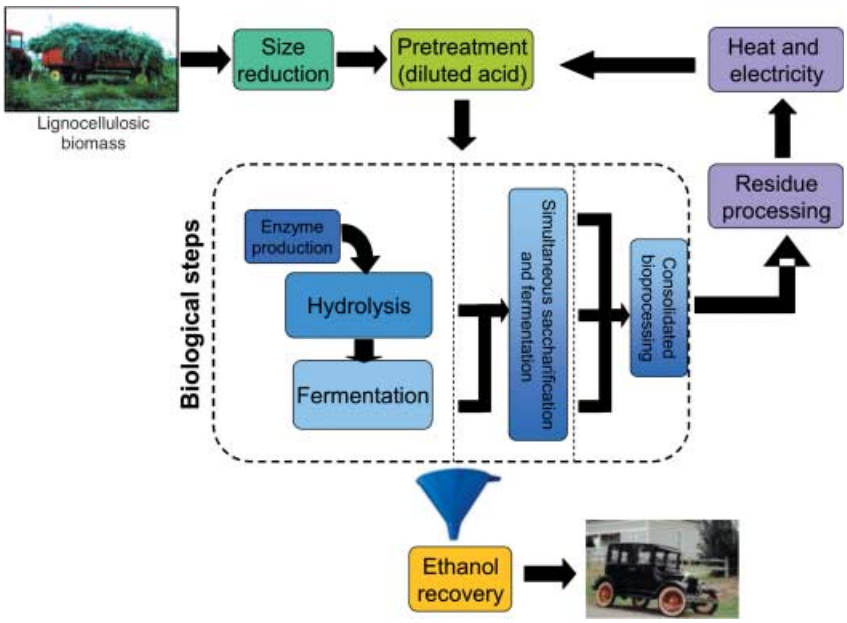

Fig. 2 Configuration of the biochemical conversion of biomass into ethanol. The original configuration of the process, total acid hydrolysis of the lignocellulose, to produce sugars was replaced by enzymatic hydrolysis. The figure shows three different configurations of the biochemical conversion. Separate hydrolysis and fermentation (SHF) is shown on the right side of the box corresponding to the biological steps. To reduce the number of reactors and decrease the inhibition of enzymes by product inhibition, hydrolysis and fermentation were combined in one step, called simultaneous saccharification and fermentation (SSF). This configuration reduces the costs of the processing of softwood by approx. 20%. A system incorporating the ability to ferment diverse sugar substrates in yeast is a variant of this process. Consolidated bioprocessing (CBP) combines the production of enzymes and the hydrolysis and fermentation of the heterogeneous sugars produced in one single step. Microorganisms capable of substrate utilization and end-product formation to fulfil the requirements of CBP do not exist at present. However, there have been advances in the improvement of naturally occurring cellulolytic organisms towards increasing tolerance to ethanol, elimination of inhibitory products, and utilization of pentoses to allow combined enzyme production and sugar fermentation.

and the other biochemical processing. Thermochemical processing defines the conversion of biomass into a range of products, by thermal decay and chemical reformation, and essentially involves heating biomass in the presence of differing concentrations of oxygen. At one extreme, heating in the presence of air leads to combustion and the release of heat that can be used in power generation or as a heat supply. The exclusion of oxygen during heating is a process termed pyrolysis, which produces various organic liquids that can be manipulated or refined to make liquid fuel. Alternatively, heating with low concentrations of oxygen leads to gasification and the production of hydrogen and organic gases, which in turn can also be converted into liquid fuels by the Fisher–Tropsch process (Fischer & Tropsch, 1926). The clear advantage of thermochemical processing is that it can essentially convert all the organic components of the biomass, compared with biochemical processing, which focuses mostly on the polysaccharides. The downside of this route to biofuels is that the start-up and plant maintenance costs are high because of the demands of high-temperature processing. Similarly, in order to operate efficiently, thermochemical processing plants require very high levels of feedstock, which necessitates the transportation of biomass over long distances, resulting in an increase in both cost and carbon footprint. From this section onwards we will focus in the biochemical processing of lignocellulosic biomass.

1. Biochemical processing

Biochemical processing involves converting biomass into sugars which can then be fermented to produce alcohols, such as ethanol or butanol, by the process outlined in Fig. 2. This type of process is attractive because the start-up and maintenance costs are substantially lower than for thermochemical plants, and biochemical processing plants can operate on a smaller scale, enabling localized feedstock production and transportation over shorter distances. Within biochemical processing, it is the saccharification, or conversion of biomass to sugars, that represents the major technical hurdle to realizing cost-effective production of liquid fuels from plant biomass by the biochemical route.

2. Saccharification – the major technical bottleneck to efficient biochemical processing

Plant cell walls are fibre-composite materials that define much of the characteristic form and function of plants. Young growing plant cells are encased in a strong but flexible cell

wall, allowing cells and organs to expand as a plant grows. In older tissues, the cell walls become substantially rigidified and are reinforced by the deposition of secondary cell walls. It is these secondary cell walls that provide mature plant tissues with their strength and resilience, forming the woody tissues which, for example, allow trees to attain their great sizes, and the stems of crops such as wheat to bear the weight of seeds produced on the plant. The major portion of plant cell walls is composed of polysaccharides and hence is potentially a rich source of sugars for fermentation.

The problem is that plant cell walls have evolved not only for strength but also for resistance to biochemical attack by living organisms. The cell wall is the first barrier between plant cells and the environment, and they are the site at which the decisive interactions with pests and pathogens occur. Even in senesced woody tissues, resistance to pests is essential if the skeletal support function of these tissues is to be maintained. Thus, plant cell walls have evolved as materials that are extremely recalcitrant to enzymatic digestion. Whilst this may represent a successful evolutionary step, it makes the release of sugars that are locked in this structure a difficult process (Wingren *et al.*, 2003).

Two approaches are applicable for the hydrolysis of plant biomass and the subsequent release of sugars for fermentation. The acid hydrolysis method, which uses acids such as sulphuric acid or hydrochloric acid, involves costly, energy-demanding steps to recover the acid used, and to condition the released sugars for fermentation (Wingren *et al.*, 2003). Alternatively, the enzymatic process, which is widely accepted to be the most efficient means of obtaining fermentable sugars from biomass, uses enzyme mixtures to achieve this (Galbe & Zacchi, 2002).

3. Enzymatic saccharification

Cellulose occurs as crystalline microfibrils formed by the association of numerous β-1,4 glucan chains. A wide range of heterotrophic organisms use cellulases to derive nutrition from plant cell walls. The commercially produced cellulases used for saccharification are in fact a cocktail of several enzymes that together convert cellulose to simple sugars, and the most commonly used ones are sourced from *Trichoderma reesei*. The two key activities in this cocktail are endoglucanases, which attack glucan chains along the microfibril surface leading to reduced polymer lengths, and exo-glucanases, which attack the polymers from their ends. The products of these two enzymes are largely cellobiose, and their activities are prone to feedback inhibition from this molecule. To overcome this potential problem, β-glucosidases, which hydrolyse cellobiose to glucose, are also included in the commercial preparations (Bayer *et al.*, 2007). Until recently, these enzymes were prohibitively expensive; however, a major research investment by the US Department of Energy into research by Novozymes and Genencor (two of the biggest industrial enzyme producers) has brought costs down by *c.* 10-fold (Percival Zhang *et al.*, 2006). In spite of these major gains, it is still the case that plant biomass requires expensive pretreatment before this material can be saccharified effectively by cellulases.

4. The need for pretreatment

As well as mechanical processing to reduce the size of the plant biomass, pretreatment is needed to allow the hydrolytic enzymes to access their substrates. To be effective, a pretreatment must deal with three issues. (1) Most of the useful sugar in lignocellulose is locked up in the cellulose microfibrils, and these themselves are highly indigestible as a result of their crystalline nature. (2) Cellulose microfibrils are embedded in a matrix of hemicelluloses, composed largely of five-carbon (pentose) sugars, and these cause a number of complications for fermentation. (3) Lignin is undoubtedly the most significant underlying feature of plant biomass, which renders it recalcitrant to digestion. This phenolic polymer essentially encases the polysaccharides of the cell walls, producing a strong and durable composite material resistant to enzymatic attack.

Several physical, chemical and enzymatic pretreatments have been developed to improve the digestibility of biomass, but the need to reduce the energy inputs, and the costs of the procedure in general, has generated consensus around the use of simple thermochemical pretreatments. Pretreatments with ammonia improve digestibility by decreasing the crystallinity of cellulose fibrils or, at high temperatures, by depolymerizing lignins and releasing matrix polysaccharides (Teymouri *et al.*, 2005). One of the most cost-effective pretreatments is the use of dilute acids (usually between 0.5 and 3% sulphuric acid) at moderate temperatures (Wyman *et al.*, 2005). This dilute acid pretreatment enables the removal of hemicelluloses and the recovery of the component sugars. Whilst lignins are not removed by this treatment, their disruption results in a significant increase in sugar yields. The efficiency of hydrolysis is increased by acid pretreatment, but this also raises the overall costs because of the need for costly equipment and post-treatment neutralization.

Following enzyme treatment, the sugars released need to be recovered and conditioned (usually concentrated) into a form suitable for fermentation into the appropriate alcohol. The end product is typically ethanol, although there has also been renewed interest in the production of butanol in recent years (Qureshi *et al.*, 2007). The major potential advantages of butanol over ethanol as a transportation fuel are that butanol is less hygroscopic than ethanol, can be mixed at higher levels with gasoline for use in conventional engines, and has a higher energy density in comparison with ethanol (Ezeji *et al.*, 2007).

Any approach for improving the process of saccharification in lignocellulose requires, or at least will benefit from, a thorough understanding of the structure and biosynthesis of

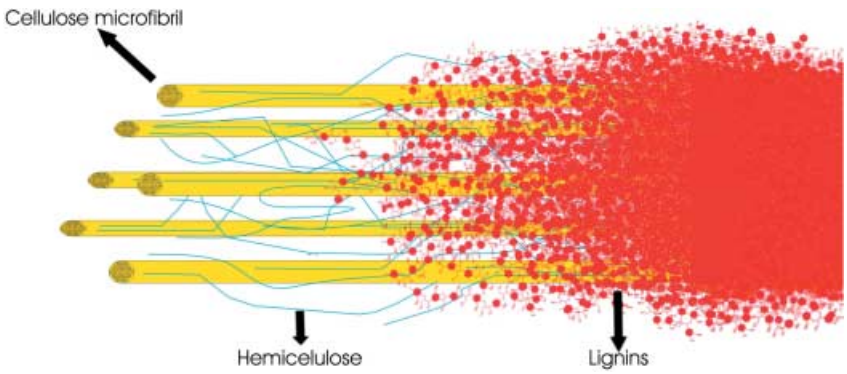

Fig. 3 Schematic representation of the generalized components of plant secondary cell walls. Cellulose microfibrils provide the structural framework of the wall and these are associated with a coating of hemicellulosic polysaccharides that hydrogen-bond to the microfibril surface and span the distance between fibrils, effectively tethering them to one another. This polysaccharide complex is effectively interpenetrated and encased by lignin, a polyphenolic polymer. In the diagram, the lignin is artificially thinned towards the left hand side of the image, simply to enable the polysaccharide components to be seen; in lignocellulosic biomass, the polysaccharides are mostly sealed up in the lignin matrix.

plant cell walls. In the following sections we will review briefly what is understood regarding the three major components of plant cell walls and indicate some of the major topics for future research.

V. General composition of plant biomass

In general, lignocellulose is made up of three major constituents, cellulose, hemicellulose and lignin, and a clear understanding of the nature and behaviour of these components will inform any progress made in optimizing their conversion into useful products. Lignocellulose is a cohesive composite material constructed around a framework of cellulose microfibrils, which perform a similar function to glass or carbon fibres in man-made composites, endowing the material with tensile strength and stiffness. In a cell wall, cellulose microfibrils are normally coated with hemicelluloses, which are polysaccharides that bind to the outer surface of microfibrils, effectively producing a hairy coat. This fibre coating acts generally to 'plasticize' the material by maintaining the distance between neighbouring microfibrils and preventing them from making direct contact, thereby maintaining flexibility in the material. This flexibility is particularly important during cell expansion, when the cell walls need to be highly extensible to accommodate often rapid and large-scale cell wall extension. In addition to their plasticizing effect, hemicelluloses, paradoxically, also appear to perform a cross-linking function, by spanning the distance between neighbouring microfibrils and partially anchoring them to one another. The degree and strength of this anchoring function appear to be modulated in a fine-tuned manner during cell expansion by a range of cell wall proteins that either cut these tethers or loosen their association with microfibril surfaces (Cosgrove, 2005).

Secondary plant cell walls (which typically dominate the composition of plant biomass) are laid down after cell expansion and form substantial thickenings and reinforcements to stabilize the structure of the cells and plant as a whole (Fig. 3 shows a diagrammatic representation of typical secondary cell wall structure). The role of hemicelluloses in secondary cell walls is not yet clear; however, recent studies in Arabidopsis have revealed that mutants with reduced secondary cell wall hemicelluloses are dwarfed, and exhibit thinner cell walls, as well as collapsed xylem vessels, indicating important functions (Brown *et al.*, 2007; Persson *et al.*, 2007). Interestingly, some secondary cell walls in woody angiosperms contain little or no hemicellulose or lignin. Tension wood is produced on the upper edge of a load-bearing stem or branch and the cells of this tissue contain a thickened layer of cell walls known as the gelatinous layer, which is composed mostly of cellulose with few other components (Pilate *et al.*, 2004). Tension wood cells contract as they dry out, thereby producing tension and reinforcing the organ mechanically, and the composition of this gelatinous layer appears to be critical to this contraction.

The polysaccharides of secondary plant cell walls are effectively sealed in a waterproof and chemically durable polymeric matrix called lignin. Lignin is a highly problematic polymer from the point of view of processing, but an exemplary evolutionary achievement. The development of lignified cell walls by land plants was a key step in enabling their preeminence in the terrestrial biosphere (Rogers *et al.*, 2005). Not only did the mechanical reinforcement of cell walls by lignin enable the development of extensive vascular systems to allow the transpiratory movement of water through plants but, by further reinforcing the mechanical properties of cell walls, this polymer may have been key in allowing plants to attain the impressive stature seen in trees. In addition to providing waterproofing and mechanical reinforcement to the cell wall, lignin forms a formidable barrier to microbial digestion.

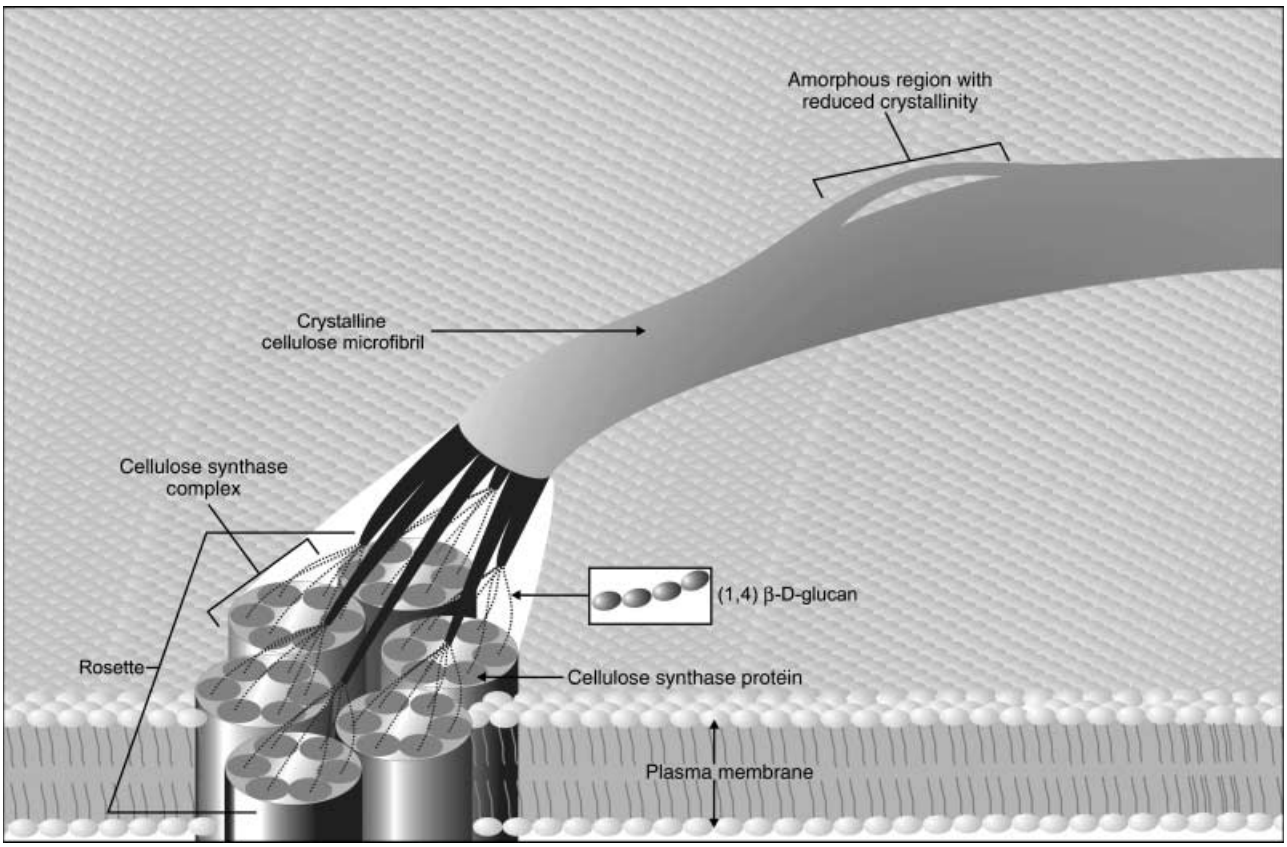

Fig. 4 Cellulose microfibrils are synthesized by large protein complexes (rosettes) embedded in the plasma membrane. Each lobe of the rosette is thought to contain six cellulose synthase catalytic sites producing cellulose chains in a coordinated fashion, giving rise to subfibrils, which come together to form crystalline microfibrils, with occasional areas of discontinuity, or amorphous regions.

1. Cellulose

Cellulose microfibrils are macromolecular structures composed of semicrystalline arrays of β-1,4 glucan chains associated with one another through extensive hydrogen bonding. Each glucose residue is inverted relative to its neighbours, resulting in a linear chain of sugar residues. This linearity allows close associations to form with neighbouring chains over long distances, resulting in a semicrystalline structure. In turn, the crystallinity of cellulose microfibrils makes them resistant to hydrolysis because the absence of water from the structure and the strong associations between glucan chains impede the access of hydrolases to the individual β-1,4 glucan chains.

The β-glucan chains of cellulose are highly insoluble (oligosaccharides with a degree of polymerization greater than 5 readily fall out of solution *in vitro*) and the ordered nature of cellulose microfibrils arises because the individual polymers in the fibril are synthesized simultaneously by the close association of numerous catalytic units (Turner *et al.*, 2007). The microfibrils are produced by large protein complexes, with a notable sixfold symmetry, found in the plasma membrane. These complexes can be visualized by scanning electron microscopy and are referred to as rosettes (Fig. 4). Each rosette has six hexagonally arranged lobes, and, because higher plant microfibrils appear to contain ~36 glucan chains in cross-section, it is thought that each lobe contains six cellulose synthase catalytic units responsible for polymerizing glucan chains from UDP-glucose available on the cytoplasmic side of the membrane. The glucan chains must coalesce in an ordered fashion on the extracellular side of the membrane complex in order to produce well-ordered microfibrils. The ordering of the glucan chains in the microfibrils occasionally falters, leading to disordered areas along the length of microfibrils, and these 'amorphous' regions are believed to serve as areas in microfibrils where hemicelluloses are more readily associated. These disordered regions are also more readily accessible to hydrolytic enzymes, and it seems likely that increasing the disordered regions in cellulose could provide a means of increasing cellulose digestibility.

Research directions for cellulose In our opinion the two main research priorities associated with cellulose and second-generation biofuels are to increase the total cellulose content in plants and to improve the digestibility of cellulose.

Increasing cellulose content Increasing the total amount of cellulose in a plant sounds superficially straightforward. However, closer inspection quickly reveals that this may only be accomplished by the up-regulation of the whole pathway of cellulose biosynthesis and carbon flow in the plant, because of the complex nature of the biosynthetic apparatus and competing demands for sugars in the plant cell. During plant development, the carbon fluxes and allocation of sugars into cellulose change in order to meet the demand of growth. A recent study involving a combined quantitative trait locus (QTL) analysis of biomass and metabolite profiling showed that growth determines global changes in metabolites rather than vice versa, underlying the importance of the developmental control of growth and cellulose biosynthesis (Meyer *et al.*, 2007). Studying the developmental control of cellulose biosynthesis might involve understanding the normal regulation of cellulose biosynthesis in the plant and identifying mutants with altered quantities of cellulose.

Whilst cellulose is present in almost all plant cell walls, it has become clear that the synthesis of primary cell wall cellulose and the synthesis of secondary cell wall cellulose are distinct processes involving separate groups of cellulose synthase genes (Johansen *et al.*, 2006). Of particular interest in this area is the study of tension wood formation. Tension wood is particularly interesting as the gelatinous layer, which is composed almost exclusively of cellulose with little lignin or hemicellulose and is thereby a rich source of potential glucose. Clearly, an understanding of the regulation of tension wood formation might provide a mechanism for the production of ectopic tension wood in plants, resulting in increased cellulose content.

Increasing cellulose digestibility A potential route to increasing digestibility lies in increasing the disordered regions in the cellulose microfibrils, and this requires an understanding of how microfibril crystallinity is controlled. Whilst the genes encoding the cellulose synthase catalytic units have now been studied extensively, studies at the protein level have proved far more difficult. The fact that cellulose synthases are integral membrane-spanning enzymes makes them difficult to isolate and study, and furthermore they appear to work cooperatively in large membrane-associated complexes, which exacerbate this problem. Researchers have, for many years, been trying to obtain plant protein preparations capable of synthesizing cellulose *in vitro*, as this would open up the possible identification of the components of the biosynthetic complex, which is generally anticipated to contain more than just the glucose polymerizing enzymes themselves (Turner *et al.*, 2007). Fortunately, there has been recent progress in this area with the first demonstration of *in vitro* cellulose biosynthesis (Lai-Kee-Him *et al.*, 2002), and this advance presents the possibility of identifying the components of the cellulose synthase complex. Other approaches are also now available for identifying components of the complex. For example, cellulose synthase mutants have been successfully complemented with epitope-tagged recombinant cellulose synthases (Taylor *et al.*, 2003), which may enable the affinity purification of associated proteins. Similarly, new advances in proteomic methods, such as those for identifying protein complexes (Hartman *et al.*, 2007), may also help shed light on the components of the cellulose synthase complex. Genes encoding proteins involved in common biochemical pathways are often under common transcriptional control and, because of this, coexpression studies using microarray data have been used successfully to identify genes involved in secondary cell wall biosynthesis, for example (Brown *et al.*, 2005; Persson *et al.*, 2005). These studies using cellulose synthase genes might also help in the identification of genes encoding other proteins from the cellulose biosynthetic complex.

2. Hemicellulose

The term hemicellulose describes a group of polysaccharides that interact intimately with cellulose microfibrils by virtue of their ability to hydrogen-bond to glucan chains. These are typically polymers of β-1,4 linked glucose or glucose-like sugars such as mannose or xylopyranose. These linear polymers tend to be insoluble and, because of this, they are usually substituted with other sugar side-chains to prevent the formation of crystalline structures, and to increase their overall solubility. Hemicelluloses bind to the outer surface of microfibrils, forming a 'hairy' coat which prevents microfibrils from directly contacting one another. These polymers can effectively link microfibrils to one another, producing a cohesive network. Xyloglucans form the major hemicellulose in many primary cell walls. However, in secondary cell walls, which predominate in plant biomass, the hemicelluloses are most typically xylans and arabinoxylans (Fig. 5).

Typically, hemicelluloses comprise between 20 and 50% of the polysaccharides in lignocellulose, and therefore contribute significantly to the potential for liquid biofuel production. Unfortunately, the yeast and bacterial species commonly used for ethanol production are not very efficient at metabolizing pentose sugars such as xylose and arabinose and, indeed, these sugars can be inhibitory to the activity of these organisms. There are two approaches to overcoming this particular problem. The first is to identify or engineer fermentative microbes that can efficiently use pentose sugars efficiently, and considerable progress is being made in this regard (Zhang *et al.*, 1995). The second is to replace the pentose-containing hemicelluloses with six-carbon (hexose) polysaccharides; this ought to be possible as hexose-based hemicelluloses serve well in a number of cell wall types; for example, mixed link glucans make up the major hemicellulose in rapidly expanding grass cells (Burton *et al.*, 2006) and the major hemicellulose of conifer secondary cell walls is a glucomannan. Hemicelluloses

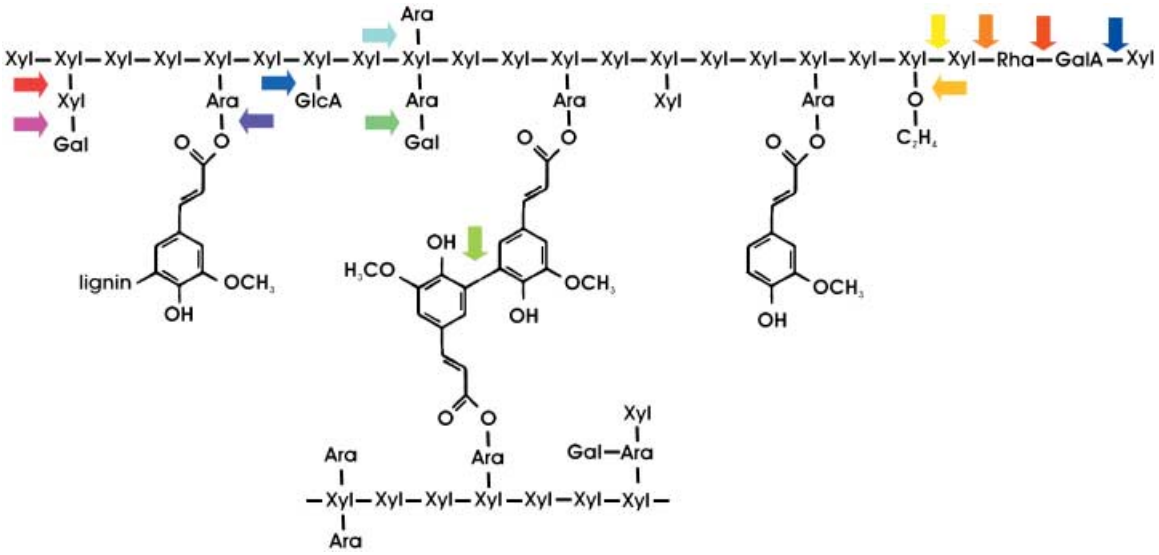

Fig. 5 Structure of arabinoxylan. The complexity of the structure is reflected in the number of different linkages present in arabinoxylans. Each arrow represents a distinctive linkage that requires an individual enzyme for its synthesis.

may have another important role in plant biomass, in that the arabinosyl residues of arabinoxylans in grasses are thought to provide the site of linkage between the lignin and polysaccharide networks (Wong, 2006) and disruption of these links should make the lignin network easier to disrupt during processing. In order to approach the problem of modifying hemicellulose content in secondary cell walls, we need a clear understanding of the biosynthesis of these polymers and the genes involved.

Hemicelluloses are biosynthesized in the Golgi system and secreted into the cell wall where they associate with the cellulose microfibrils. The proteins and genes directly responsible for hemicellulose biosynthesis have begun to emerge in the post-genomic era, but much remains unknown. Xyloglucans are the most studied in this context, and this polysaccharide requires a minimum of four distinct glycosyl transferase activities for its synthesis. Two of these activities have been characterized (Perrin *et al.*, 1999; Madson *et al.*, 2003) and candidate genes have been identified for the other two (Cavalier & Keegstra, 2006; Cocuron *et al.*, 2007). Much less is known in the case of xylans and arabinoxylans, although several Arabidopsis mutants with reduced xylan levels have been isolated recently from screens of candidate genes identified by co-expression studies with secondary cell wall cellulose synthase (*CesaA*) genes (Brown *et al.*, 2005; Persson *et al.*, 2005).

In grass cell walls, the arabinosyl residues of arabinoxylans are often esterified with ferulic acid residues that may be coupled in dimers, effectively crosslinking neighbouring polymers to one another (Lindsay & Fry, 2007). This might even serve as the site of direct connections between polysaccharides and lignin, and, as such, these polysaccharides may have a direct influence on the integrity of the wall polymeric network, and modification of these connections might lead to a more easily saccharified cell wall. Hemicelluloses such as xylans and arabinoxylans are often acetylated, and this modification serves to decrease polysaccharide solubility, and this in turn may also have an impact on the subsequent saccharification of these materials. Hence genes encoding both feruloyl and acetyl transferases are current targets for research (Mitchell *et al.*, 2007).

3. Lignin

Lignin is undoubtedly the most significant underlying feature of plant biomass, which renders it recalcitrant to digestion. This phenolic polymer essentially encases the polysaccharides of the cell walls, producing a strong and durable composite material resistant to enzymatic attack. It is the disruption of lignin and dissolution of cellulose crystallinity that represent the main targets of pretreatments before enzymatic saccharification. Fortunately, lignin biosynthesis is probably the best understood aspect of plant cell walls because it has seen considerable research investment in recent decades. The reason for the emphasis on lignin research is that it has been seen as a commercially important target for many years, largely because lignin disruption is a major cost-contributor to the process of making paper (Halpin, 2004). Most genes encoding lignin biosynthetic enzymes have been identified and investigated at the genetic and reverse genetic levels in a range of plant species. This has led to the identification of strategies for modifying lignin content and structure in a manner that makes it easier to disrupt during pulping with minimal impact on field performance in forestry trees such as poplar and *Eucalyptus* (Baucher *et al.*, 2003).

Similarly, the role of lignins in limiting the digestibility of forage crops by domestic animals has been investigated extensively, and more digestible, lignin-modified forage grasses and maize have been taken forward for commercial development (Chen *et al.*, 2003). It seems likely that the issues determining wood pulping and forage digestibility will be closely related to those determining saccharification, and indeed recent studies have shown that more digestible, lignin-modified alfalfa (*Medicago sativa*) varieties are also more readily converted to sugars *in vitro* using industrial cellulases (Chen & Dixon, 2007). Similarly, several QTLs with significant effects on forage digestibility in maize have been identified, some of which clearly involve altered lignin (Guillaumie *et al.*, 2007). In this context, brown midrib mutants in maize and sorghum (*Sorghum bicolor*) have also been investigated for digestibility and saccharification.

Lignin research directions Given the availability of lignin-modified plants and mutants, a comprehensive and systematic analysis of saccharification in cell walls from such plants will prove highly valuable in identifying optimal approaches to modifying lignin for liquid biofuel applications. Although, compared with polysaccharide biosynthesis, lignin biosynthesis is a well-studied subject, it is important to note that a number of substantial holes in our knowledge remain. In particular, the mechanism by which monolignols are exported from the cytoplasm to the cell wall remains mysterious. It is generally held that monolignols are glycosylated before translocation across the membrane and subsequently de-glycosylated before polymerization by free radical chemistry in the wall (Boerjan *et al.*, 2003). There is, however, little evidence to support this hypothesis and this is a topic in need of renewed research effort. Another notable gap is that most lignin research has been carried out in dicot species and the complete pathway has yet to be fully characterized in grass species, which, given the suitability of grasses as dedicated biomass crops, is an area in need of attention.

VI. Overcoming the saccharification barrier

From the biological perspective, there are two major research approaches that can, and are, being adopted to deal with the saccharification issue. One approach involves improving the plant biomass feedstocks in a way that optimizes their conversion to sugars. This requires the identification of key genetic loci for optimal performance, with regard to saccharification, which can then be used for crop improvement by genetic engineering or as markers in conventional breeding. The second approach is the discovery and development of new tools for efficient cell wall disassembly *in vitro*, that is to say the discovery and optimization of enzymes for processing and pretreatment.

1. Improving biomass feedstocks – choosing the crop

The sections preceding this one have outlined some of the molecular features of cell walls that are likely to influence saccharification potential. Improving plant materials as feedstocks for second-generation biofuels could also involve the deliberate targeting of particular polymers and bonds in the cell wall using genetic modification or marker-based plant selection. However, another key question regards the choice of crops for this type of research. One possible answer is to choose the most appropriate biomass crop for the agricultural context in which the desired biomass will be grown, yet even this is not obvious as the best choices for dedicated biomass crops have yet to be made. Perennial crops are generally favoured over annuals as biomass crops, because they tend to have a higher annual net photosynthetic capacity (as a result of longer canopy duration), and do not require planting every year. These features result in a more favourable proportion of energy going into production versus that released for use as fuel. The exact crop of choice will depend on the environment to be used for production. Amongst species being actively considered as dedicated biomass crops in Europe and North America are *Miscanthus* × *giganteus* (Atienza *et al.*, 2003; Heaton *et al.*, 2004), switchgrass (Wu *et al.*, 2006), and short-rotation coppice trees such as willow (Hanley *et al.*, 2002). Many of these plants have seen little or no domestication and resources for breeding or genetically modifying some of these plants are still at a very early stage and will require considerable effort. For example, *Miscanthus* × *giganteus* is an extremely productive C_4 perennial grass, favoured by many as a biomass crop, but it is a sterile polyploid hybrid for which no efficient genetic transformation protocols have yet been published.

Genetic engineering approaches to modifying saccharification potential are attractive, as molecular changes can theoretically be carefully targeted without any impact on field crop performance, as has been demonstrated by lignin modification for paper pulping applications (Pilate *et al.*, 2002). In addition to targeting the lignin biosynthetic pathway, a number of other approaches are being considered to improve biomass processing, such as producing cellulases directly in the seeds of transgenic plants (Hood *et al.*, 2007).

An alternative approach to genetic engineering for improved saccharification is to take a more empirical approach and screen directly for genetic loci that influence saccharification. Once identified, the effects of such loci on the cell wall composition and structure can be investigated in detail, and the responsible genes identified and used in targeted breeding programmes. These approaches require the development of appropriate assays for saccharification that can be applied in a sufficiently high-throughput manner. The availability of recombinant inbred lines and mutant populations are also required and these resources require establishment for many dedicated biomass crop species. Alternatively, model species are often better suited for gene discovery research as they are typically chosen because they have smaller genomes, simpler genetics and shorter life cycles.

Over recent decades, we have built up an increasingly refined understanding of the fundamental properties of plant

cell walls and their biosynthesis. The possibility of employing advanced genomic and transcriptomic approaches in model plants has taken the field forward at an increasingly rapid rate, most particularly in *Arabidopsis thaliana*, for which the range of genetic tools and electronic databases remains unrivalled. Although Arabidopsis continues to be the best general model plant species available as a herbaceous annual, this plant falls short on specific points of structure and development as a model for woody perennials such as willow. In this case, poplar has been developed as a model species with extensive molecular genetic resources, and a fully sequenced genome (Tuskan *et al.*, 2006).

Similarly, there are clear shortcomings in the use of Arabidopsis as a model for grass species. These arise because Arabidopsis is evolutionarily quite distant from the grasses, and whilst as angiosperms they share much in common, they differ from one another in a number of distinct ways, especially in their cell wall composition. Grasses and dicots differ substantially in hemicellulose composition as well as in cell wall phenolics (Carpita & Gibeaut, 1993).

2. *Brachypodium distachyon*, a model temperate grass species

Although the rice genome sequence has been available for several years, the genetic and genomic resources for this species have been rather slow to develop. In addition, the growth requirements for rice make it a difficult species to work with in nontropical climates. For these and other reasons, there has been a general recognition of the need for a good model temperate grass species. In recent years this has culminated with the promotion of *Brachypodium distachyon* to fulfil this role. This small, weedy, temperate grass is a suitable model species because it is closely related to major crop cereals such as wheat, barley (*Hordeum vulgare*) and oat, as well as to some of the perennial grass species that are being investigated as potential biomass crops. Importantly, this plant has a small genome (twice the size of Arabidopsis) and a short life cycle, and can be genetically transformed with relative ease. As a result of these features, the entire *B. distachyon* genome has been sequenced and is being assembled at the time of writing this review (http://www.jgi.doe.gov/sequencing/why/CSP2007/brachypodium.html), and other genomic and genetic resources are expected to become available rapidly (http://www.brachypodium.org).

VII. Tools for cell wall disassembly

In this section we will briefly consider some of the possibilities for improving lignocellulose saccharification *in vitro* using biological tools (mostly enzymes). This is not directly a plant-related topic because most of the enzymes used come from microbial species. Dramatic improvements have been made to industrial cellulase cocktails in recent years, thanks to substantial funding from the US government and sustained research by major enzyme producers (Percival Zhang *et al.*, 2006), the result of which is that cellulase costs are a fraction of what they were a decade ago. Although currently it is the cost of pretreatment that dominates the expense of cellulosic ethanol production, enzymes still represent a major fraction of the costs of cellulosic ethanol.

There is widespread appreciation that there remains great potential for the discovery of novel enzymes to be used in tackling the saccharification barrier. Such enzymes may either be used (through transgenesis) to increase the predisposition of plant biomass for saccharification by modifying cell walls during plant growth, or be employed directly during processing *in vitro* to overcome the recalcitrance of lignocellulose to cellulases. Whilst plant biomass requires costly pretreatment to allow efficient saccharification in the industrial context, there are numerous examples from nature where lignocellulose is successfully converted into useful sugars without the use of costly pretreatments. Notable examples are the composting of plant waste in the environment, and the digestive systems of a limited number of organisms able to subsist on a lignocellulose-rich diet, such as termites and wood-boring insects, arthropods and molluscs. In almost all such cases, lignocellulose is degraded through the action of complex microbial communities either free-living or living symbiotically in animal hosts. Less than 1% of microbes from any environment can be cultured (Ferrer *et al.*, 2005) and, because of this, there is recognition that culture-independent methods for gene discovery hold the key to opening the unexplored potential of the microbial world. Consequently, industrial enzyme companies are turning to metagenomics-based bioprospecting to find new and more efficient enzymes. Metagenomic approaches to bioprospecting involve sequencing DNA isolated from wild microbial populations and using sequence-similarity searching, coupled to high-throughput protein expression and activity studies. One of the most well publicized of these discovery programmes is that being carried out by the US Department of Energy (US DOE) and Verenium Corporation working on the termite hind gut (http://www.jgi.doe.gov/sequencing/why/CSP2006/termitegut.html).

VIII. Prospects for biofuels

In line with growing worries over climate change and sustainability, biofuels have received generally positive support in the popular press. However, growing concerns over first-generation biofuels in terms of their impact on food prices and the environment have led to an increasingly bad press in the last year. The struggle of 'land vs fuel' will be driven by the predicted 10 times increase in biofuels until 2050. The unfortunate effect is that biofuel is starting to generate resistance, particularly in poor countries, and from a number of activist non-governmental organisations with environmental agendas. This is highly unfortunate as it is clear that liquid

biofuels hold the potential to provide a more sustainable source of energy for the transportation sector, if produced sensibly. As the replacement of fossil fuels will take place irrespective of these concerns, the way to avoid the negative effects of producing biofuels from food supplies is to make lignocellulosic-derived fuels available within the shortest possible time. This process involves an unprecedented challenge, as the technology to produce these replacement fuels is still being developed. However, the immediate use of first-generation biofuels involves putting in place logistic changes to use biofuels (engine modification, distribution, production plants, etc.). This commitment to biofuels in the present will make the transition to the second generation of biofuels more economically convenient.

The contribution of biofuels to solving the present energy crisis relies on a technological breakthrough to meet the projected demand. The latest developments in the areas of enzyme production and cell wall biology bring the goal of sustainable biofuel production closer to realization. Moreover, the current investments in research and development by both governments and private companies provide the scope for a fast learning curve in the whole area. Indeed, in the last year over 1 billion dollars has been committed for research into second-generation biofuels in the US alone (http://genomicsgtl.energy.gov/centers/, http://www.ebiweb.org/).

As well as carrying out the research needed to bring this enterprise to fruition, it is important that scientists from a range of disciplines, including those studying the environmental impact of biofuels and those working on improving the process of making them, communicate as loudly and clearly as possible with the public at large. We need to promote greater sustainability and responsibility in the way we use the resources of the planet, but it is equally clear from past experience regarding genetically modified crops that it is imperative to inform the rest of society in as clear a way as possible about the potential benefits of this move, as well as the perils of not taking action.

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