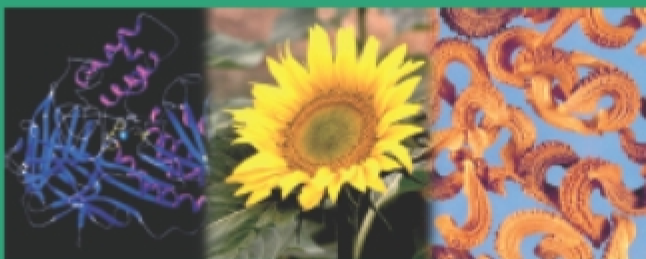
CELL WALL SACCHARIFICATION



epobio

Realising the Economic Potential of Sustainable Resources
- Bioproducts from Non-Food Crops

CELL WALL SACCHARIFICATION

Outputs from the EPOBIO project

November 2006

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cplpress

Science Publishers

EPOBIO: Realising the Economic Potential of Sustainable Resources - Bioproducts from Non-food Crops

© November 2006, CNAP, University of York

EPOBIO is supported by the European Commission under the Sixth RTD Framework Programme Specific Support Action SSPE-CT-2005-022681 together with the United States Department of Agriculture.

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Published by:

CPL Press, Tall Gables, The Sydings, Speen, Newbury, Berks RG14 1RZ, UK
Tel: +44 1635 292443   Fax: +44 1635 862131   Email: orders@cplpress.com
Website: www.cplbookshop.com

ISBN 10: 1-872691-04-8   ISBN 13: 978-1-872691-04-6

Printed in the UK by Antony Rowe Ltd, Chippenham

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Executive Summary

EPOBIO is an international project to realise the economic potential of plant-derived raw materials by designing new generations of bio-based products that will reach the market place 10-15 years from now. At a Workshop held in Wageningen in May 2006 a wide range of experts considered the Flagship theme of plant cell walls in relation to biorefining. They identified the need to improve the efficiency with which lignocellulosic plant cell walls, the most abundant renewable resource on earth, can be converted into sugars and other useful bioproducts through biorefining, as the first target for EPOBIO to consider. This report sets out the conclusion of a detailed literature review and also takes account of inputs from international scientists and industrialists.

Biorefining is the production of chemicals, materials, fibres, products, fuels or power from agricultural/forestry raw materials. First generation biorefineries use simple feedstocks such as sugar, starch or vegetable oil, but second and third generation biorefineries are already in development and will use biomass feedstocks that largely consist of lignocellulose cell walls from plant-based feedstocks. The biorefinery is already recognised to have a key role to play in the production of renewable fuels including bioethanol and biodiesel. Significantly, future generations of biorefineries will be integrated, zero-waste systems producing many bioproducts and materials from a diverse range of feedstocks.

Cost-effective, efficient conversion of plant cell walls into their components is key to realising the full potential of the biomass lignocellulose feedstock. Plant cell walls have evolved to resist breakdown, whether from mechanical or chemical forces or from microbial attack. This resistance to breakdown is a massive bottleneck for the development of second generation biorefineries. Understanding the complexity of plant cell walls and ways in which sugars can be more efficiently released from the walls (saccharification) were considered to be a major priority for EPOBIO.

From a policy and regulatory perspective, the development of efficient and cost-effective biorefineries is important for a number of reasons. Biorefineries can make a positive contribution to the delivery of international targets and governmental commitments for reductions in greenhouse gas emissions whilst also addressing energy supply issues. Innovation directed to the development of new generations of more efficient biorefineries will deliver a major improvement in the level of the greenhouse gas emission reductions achieved. Biorefineries are a key strategy of the Knowledge-Based Bio-Economy (KBBE), delivering renewable and sustainable products able to compete with existing fossil-derived products.

The production of biofuels in biorefineries and reducing dependence on fossil reserves are driven by a number of strategic imperatives including the price, finite nature and security of supply of fossil oil. Other drivers include the detrimental environmental impact of fossil-derived fuels and mineral oils compared with the renewable and sustainable nature of plant-derived alternatives. There are also important regulatory drivers such as the indicative target in the EU of 5.75% biofuels by 2010, a target that is under review with further proposals likely. In the US, policy initiatives include the Energy Action Plan, mandating an increase in the use of bioethanol and biodiesel, and the Advanced Energy Initiative, promoting the development of practical and competitive methods for the production of bioethanol from lignocellulose.

There is also increasing concern about the environmental impact of the expansion of oil palm, soybean and sugar cane cultivation for biofuels leading to deforestation in Indonesia, Malaysia and Brazil. The future development of second generation lignocellulosic biorefineries in Europe and the US affords the potential to track and evidence environmental impacts and benefits, increase biofuel production in those regions whilst, in parallel, addressing environmental concerns about the use of imported material.

In the context of a Common Agricultural Policy that has cut the link between subsidy and production and brought a new focus on the market, biorefineries will provide an

additional outlet for the agriculture sector, especially in the newer Member States. Structural funds could readily be used to support biorefinery investment in those countries as well as in less prosperous Objective 1 regions of the EU. New income opportunities are linked to the potential for diversification in agriculture. New commercial markets will not only help the viability of farming but will also encourage sustainability and develop the wider rural economy and infrastructure.

Biorefineries are also highly relevant to policies that aim to support developing countries. Biorefineries in developing countries could readily deliver social and economic benefits through the production of biofuels and energy for local use integrated with bioproducts for export. Clear technical standards would need to be set to ensure the market and supply chains develop on a sound commercial basis.

An important aim of biorefining is to maximise the value derived from the biomass feedstock. The harsh chemical and physical treatments currently used in biorefineries involve a significant energy use and can often lead to a loss of value in bioproducts. New processes that protect the by-products and enhance their value will support wealth creation and add further value to agricultural outputs. For efficient biorefining the component parts of the biomass must be released in a way that protects their value. To minimise input costs biorefineries will also need to be able to use a wide range of feedstocks.

The composition and molecular organisation of plant cell walls vary between feedstocks and are responsive to environmental change. The report identifies that there is a need to develop molecular and analytical tools to characterise the diverse range of biomass feedstocks and, in parallel, design novel high throughput assays for their digestibility. Research into cell wall pre-treatment is also needed. The use of cellulases is fundamental to efficient biorefining and there is a need to further optimise cellulases. Also, novel hydrolases need to be identified to improve breakdown of the complex and highly resistant plant cell walls.

The scale of the work needed is both significant and international requiring multidisciplinary collaboration. A single integrated project spanning the diverse research areas would ensure continuous feedback and a full exchange of know-how and materials.

1 INTRODUCTION

In May 2006 the first EPOBIO workshop was held in Wageningen, The Netherlands. Experts in the areas of plant cell wall, plant biopolymer and plant oil research participated in the workshop, and breakout sessions were held to discuss and identify research needs to underpin the development of novel products from non-food crops within the next 10 – 15 years (EPOBIO report 2006). The identified research needs are now being investigated in the EPOBIO project and a series of reports will be produced giving recommendations for future research directions, thereby supporting policy makers in decision finding.

The first research need investigation of the Plant Cell Walls Flagship is aimed at improving the efficiency and reducing the cost of a key generic process in cell wall biorefining. This process is saccharification, the conversion of input biomass into C5/C6 sugars - essentially, the digestion of plant cell walls. This project underpins the development of the subsequent work for the Flagship, since the nature of the processes chosen for saccharification determines the range of materials and value products that can also be derived from the input biomass.

Different approaches can be taken for making saccharification more efficient and cost-effective, for example pre-treatment technologies and process integration in biorefineries can be improved (Mielenz 2001).

The present research need investigation, however, focuses on biological solutions to this problem, which include the improvement of biorefinery feedstock through better understanding of cell walls, and improvement of hydrolytic enzymes (EPOBIO report 2006). Three research areas related to this will be reviewed in detail and the research needs defined. These are i) development of analytical tools for characterising cell walls of biorefinery feedstock, ii) design of assays for monitoring cell wall digestibility and products formed iii) identification of novel hydrolytic enzymes that can degrade cell walls more efficiently.

2 CELL WALL BIOREFINING

A significant proportion of the plant biomass on Earth is made up of cell walls (Poorter and Villar 1997). These are composed of up to 75% of sugar polymers. Other cell wall components are lignin and proteins. In the context of biomass utilisation and biorefining, cell walls are therefore often referred to as lignocellulosic material. Plant cell walls represent an abundant resource of sugars (Zoebelein 2001). These sugars can be used as raw materials for the manufacture of various products such as ethanol, butanol, acetic acid, citric acid, levulinic acid, lactic acid or furfural (Willke and Vorlop 2004, US Department of Energy 2004, Lichtenthaler 2006). A key step for using these sugars is the degradation of cell walls into sugar monomers. This process is called saccharification.

Currently, there is great interest in using lignocellulosic biomass as raw material for the manufacture of bio-based products, especially ethanol (Schubert 2006). Manufacture of bio-based products is envisioned to take place in integrated processing units called biorefineries. The US Department of Energy defined the term biorefinery as: "an overall concept of a processing plant where biomass feedstock is converted and extracted into a spectrum of valuable products" (US Department of Energy 1997). The concept is analogous to that of today's petrochemical refineries (National Research Council 2000, Kamm and Kamm 2004). Several companies are planning to build commercial biorefineries, mainly for the purpose of producing cellulosic (lignocellulosic) ethanol. Research in this area is very intense and pilot plants for the validation of the production process are operating. However, no commercial cellulosic ethanol plant has been built to date (Table 1).

Research in the area of cell wall biorefining is mainly focused on cellulosic ethanol production. The use of biofuels to replace non-renewable, petroleum-derived fuels is promoted in Europe and the United States by Directive 2003/30/EEC and the US Energy Policy Act of 2005, respectively (The National Non-Food Crops Centre 2005, Demirbas and Balat 2006, Gray et al. 2006, Ragauskas et al. 2006). In Europe 5.75% of all petrol and diesel for transport, calculated on the basis of energy content, shall be replaced by fuels produced from biomass, including ethanol, by 31

December 2010. In the US, 28.4 billion litres (7.5 billion gallons) of biofuels have to be blended into gasoline by 2012, 113 billion litres (30 billion gallons) by 2020 and 226 billion litres (60 billion gallons, i.e. 30% of the current US gasoline consumption) by 2030. In 2004 the US produced about 12.8 billion litres (3.4 billion gallons) of ethanol from corn starch and used about 11% of the US corn harvest (Hammerschlag 2006, NRDC 2006). It is estimated that the US has the capacity to produce 49.2 billion litres/year (13 billion gallons/year) of ethanol from corn starch, and further increases in ethanol production will have to come from other feedstock, such as plant cell walls (Gray *et al.* 2006, National Corn Growers Association 2006).

Several reasons underpin the current interest in the production of bio-based products from lignocellulosic materials (US Department of Energy 2006a). Fossil resources are limited and non-renewable. Mineral oil especially, which serves as raw material for fuel and chemical production, will become scarce during the next decades (Campbell and Laherrere 1998, MacDiarmid and Venancio 2006). The increasing prices will probably prohibit extensive use of this resource in future. Many countries are seeking alternative energy sources that will increase their security of energy supply.

As mentioned above, bio-based products will be produced in biorefineries, which will be built in close vicinity to the feedstock and therefore mainly in rural areas. Rural economies might therefore benefit through the creation of new jobs and higher incomes.

The production of bio-based materials, especially biofuels, will also provide positive environmental benefits such as reduced carbon dioxide emissions (Farrell et al. 2006, Wu *et al.* 2006). When fossil fuels such as oil are burned, carbon dioxide (CO_2) is released into the atmosphere, accumulates and contributes to the greenhouse effect (IPCC 2001). Carbon dioxide is also released when biofuels are burned. However, the photosynthetic growth of new biomass binds the carbon dioxide released during biofuel use. Fossil carbon dioxide emissions from ethanol produced from cell walls have been calculated to be 85% lower than those from gasoline (US Department of Energy 2006a).

Table 1 Plants for conversion of lignocellulosic biomass to ethanol in the European Union, North America, Japan.

Name (REF)	Plant type	Location	Feedstock	Process	Status
Etek plant - NILE project. (NILE Project 2006, ETEK 2006)	Pilot	Sweden	Spruce saw dust	Enzymatic or dilute acid saccharification	2004; 2 mt/day, 0,1-0,2 Mio litres/year, feasibility study for demonstration plant underway
Abengoa plant (Abengoa Bioenergy 2006)	Commercial	Spain	Barley and wheat straw	Simultaneous saccharification and fermentation (SSF)	Under construction, production start planned for autumn 2006, 70 mt/day; 5 Mio litres/year
Iogen plant (Iogen 2006)	Demonstration	Canada	Straw	Enzymatic saccharification	2004, 40 mt/day, 3 Mio litres/year, feasibility study for commercial plant underway
Bluefire Ethanol (Bluefire Ethanol 2006a)	Commercial	USA	Municipal waste	Acid hydrolysis (Arkenol process)	Construction to begin Spring 2007 (30 Mio litres/year). Begin of production planned for 2009.
Celunol (Celunol 2006)	Pilot	USA	Bagasse, energy cane, short rotation wood eg poplar, cottonwood, municipal waste	SSF	Pilot plant: October 2006 (0.2 Mio litres/year). Construction of demonstration plant to begin in October 2006 (6.4 Mio litres/year)
Izumi - JGC Corp. (Bluefire Ethanol 2006b)	Pilot	Japan	Wood chips	Concentrated Acid hydrolysis (Arkenol process)	Since 2002 (0.1 Mio litres/year)
NREL (NREL 2006a, b)	Pilot	USA	Various	Various	Various
IBUS plant (IBUS 2006)	Pilot	Denmark	Straw	Enzymatic saccharification	Old pilot plant is dismantled. Two pilot plants will be rebuild (January 2007) (1 mt straw/day; 40 mt straw/day)

2.1 Strategic vision and research activities

2.1.1 Strategic vision

The strategic visions for developing biorefineries and producing biofuels, especially ethanol, are strongly linked. Research in the area of cellulosic ethanol production will contribute to advancing the development of cell wall biorefineries that will in future produce various bio-based products, which could be butanol, lactic acid and others (US Department of Energy 2004).

The European strategy for biofuels will therefore also impact on the development of biorefinery technology. The European Commission has identified seven principal points that constitute the EU strategy for biofuels (European Commission 2006). One of these points is the support for research and development for biofuels. To quote the EU strategy:

"The Commission will

- *In the 7th Framework Programme continue its support for the development of biofuels and strengthening the competitiveness of the biofuel industry;*
- *Give a high priority to research into the "bio-refinery" concept – finding valuable uses for all parts of the plant – and into second-generation biofuels;*
- *Continue to encourage the development of an industry-led "Biofuel technology platform" and mobilise other relevant technology platforms;*
- *Support the implementation of the Strategic Research Agendas prepared by these technology platforms."*

The European Technology platforms have been established to focus on strategic issues in relation to required technological advances and to define medium to long term research and technological developmental objectives in their area (CORDIS 2006). Technology platforms that also stress the importance of the biorefinery concept are "Sustainable Chemistry Technology Platform" (SUSCHEM 2006), "Forest-based Sector Technology Platform" (Forest-based Technology Platform

2006), “Plants for the Future Platform” (Plants for the Future Technology Platform 2006) and “Biofuels Technology Platform” (Biofuels Technology Platform 2006). These technology platforms have developed or are developing vision documents and strategic research agendas summarizing the key challenges and opportunities faced today. In all of these documents and the vision paper of the “Biofuels Technology Platform”, the importance for utilizing bio-based products from non-food biomass and the need for research related to the biorefinery concept is emphasised. Especially in relation to the production of biofuels a pressing need for research in the area of cell wall conversion has been identified by the Biofuels Research Advisory Committee in 2006 (Biofrac report 2006). In Europe ethanol is currently produced from sugar or starch of sugar beets or grains. Together with biodiesel made from oil crops or waste fat, these fuels are called 1st generation biofuels (Biofrac report 2006). The 2nd generation biofuels are expected to be on the market after 2010 and they will mainly be produced from lignocellulosic biomass. These biofuels include ethanol, and synthetic biofuels, such as synthetic biodiesel, biomethanol, biodimethylether or synthetic natural gas (Biofrac report 2006). Research for developing these fuels has to be intensified in order to deploy them after 2010.

2.1.2 Previous research activities on cell wall hydrolysis

Interest in saccharification of lignocellulosic raw materials has a long history. In 1819 it was discovered that wood can be degraded to yield sugar by an acid treatment, and already by 1898 the first commercial processes were developed (Sheehan and Himmel 1999). The yields of these processes, however, were very low (Kamm et al. 2006a). Until World War II many plants using acid catalysed hydrolysis to produce ethanol from cell walls (mainly wood), were operated in Germany, Russia and the USA (Katzen and Schell 2006). At the end of World War II cheap oil became available and ethanol and other products were manufactured from this abundant raw material. The interest in further development of conversion techniques for lignocellulosic raw materials declined. During the oil crisis of the 1970s, however, interest in this field grew as alternatives to petrochemicals were sought. Studies that were carried out in the early 1980s showed that the conversion

of biomass to ethanol by certain acid-catalysed processes was economical, however, the potential of enzymatic hydrolysis to achieve higher yields, led to more research in the area of enzyme-based processing (Hall *et al*. 1956, Mandels and Sternberg 1976, Wright 1988, Coombs 1996). Compared to the long-known processes of acid-catalysed hydrolysis this relatively new technology offered huge potential for improvements. Several research programmes were initiated in the US and in Europe to advance this technology. An overview about the recent research activities up to 1995 in Europe and the US is given in a bioconversion assessment study published by the European Commission (Coombs 1996).

A more recent research activity in this field was the project "Technological improvement for ethanol production from lignocellulose" (TIME) that was completed in October 2005 (TIME 2006). The overall aim of this project was to improve the technology of the lignocellulose to ethanol process. Specific aims were optimisation of pre-treatments, identification and production of novel hydrolases, and process optimisation.

2.1.3 Current research activities

2.1.3.1 European Union

One major research project with the aim to develop cost-effective production of ethanol from lignocellulosic biomass is funded within the 6th Framework Programme of the European Union (Table 2). The Integrated Project "New Improvements for Lignocellulosic Ethanol" (NILE) runs from October 2005 to September 2009 and has a total budget of 12.8 M€, of which 7.7 M€ will be contributed by the EU (NILE 2006). NILE aims to develop cost-effective production of ethanol from lignocellulosic biomass. The project includes seven work packages (WP): WP1 Enzymatic Hydrolysis, WP2 Ethanol Production, WP3 Process Technology, WP4 Development Strategy, WP5 Evaluation of Bioethanol, WP6 Dissemination & Training, WP7 Project Management. In relation to this EPOBIO research need investigation, the NILE project is of importance, especially the programme of the WP1 Enzymatic Hydrolysis.

Table 2 Recent research projects in relation to cell wall saccharification funded by the European Commission.

Project name	Duration	Funding	Reference
New Improvement of Lignocellulosic Ethanol (NILE)	2005-2009	12, 6 Mio€ (7,7 Mio€ (EC)), FP6	NILE (2006)
Technological Improvement for ethanol production from lignocellulose (TIME)	2002-2006	4,3 Mio€ (2,6 Mio€ (EC)), FP5	TIME (2006)

This WP will search for new enzymes and aims to identify stable and efficient cellulases and related enzymes for saccharification. In addition, new fungal binding modules that improve targeting of enzymes to the substrate will be searched for. Also, additional enzymes that can enhance cellulose accessibility will be tested. The hydrolytic performance of selected enzymes will also be improved by directed evolution. New, highly efficient strains of *Trichoderma reesei* for the production of enzyme mixtures will also be engineered. Large amounts of enzymes will be produced for use in pilot plant scale.

2.1.3.2 United States of America

Also in the US, the need for further investment in research areas relating to biomass utilisation has been recognized (Renewables Vision 2020 Executive Steering Group 1999). As mentioned above, bioethanol production from corn starch cannot satisfy the requirements of the US Energy Policy Act of 2005, and bioethanol will have to be produced from other biomass, such as cell walls. The US Department of Energy (DOE), therefore, has established the Biomass Program, aiming at reducing the dependence on foreign oil by developing biomass-based liquid fuels and to foster the domestic biomass industry. Recently, the DOE announced a further investment in this research area. The DOE will establish up to two new bioenergy research centres. Major emphasis is on developing cost-effective means to produce ethanol from cell walls of non-food crops. Each of the centres will be funded for up to US$125 million over a period of five years (US Department of Energy (2006b, c).

3 SACCHARIFICATION – A KEY PROCESS FOR CELL WALL BIOREFINING

Saccharification, the degradation of cell wall sugar polymers to their sugar monomers, is a key process in cell wall biorefining. A simplified scheme of cell wall biorefining is given in Figure 1.

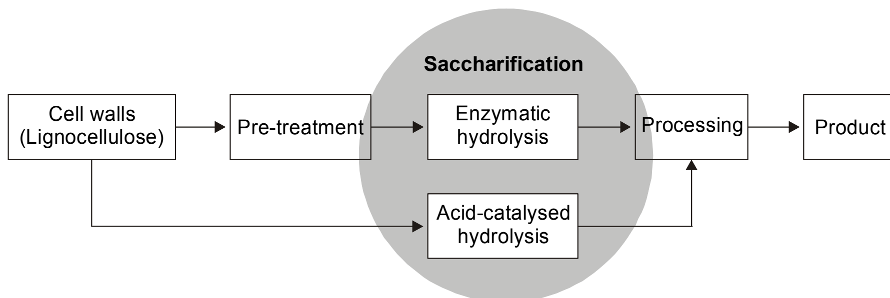

Figure 1 Generalised lignocellulosic conversion process.

Biomass does not have to be pre-treated if acid-catalysed saccharification is applied (Figure 1), however, as outlined in Chapter 2, despite this advantage compared to enzymatic saccharification, acid-catalysed saccharification is still not cost-competitive (US Department of Energy 2006a). Many researchers believe that enzymatic saccharification is a more promising technology. However, this technology is also currently not cost-competitive. The reason for this is the recalcitrance of the cell walls. They have naturally evolved to resist enzymatic, chemical and physical breakdown, precisely the processes needed for saccharification. Efficiency of cell wall saccharification is affected by many factors, for example feedstock (cell wall) characteristics, hydrolysis conditions including the mixture and type of enzymes used, and pre-treatment technology (Mansfield *et al*. 1999). To achieve the best possible saccharification efficiency for a given feedstock, pretreatment and hydrolysis conditions have to be optimised (US Department of Energy 2006a). So far it is not understood in detail, how feedstock characteristics inhibit the saccharification process (Mansfield *et al.* 1999). In addition, our understanding of the mechanisms of enzyme-catalysed hydrolysis is limited

(Mansfield *et al.* 1999). Better understanding in these areas is needed to select and design feedstock with cell wall characteristics that are favourable for bioconversion and to identify and engineer more effective hydrolases.

This chapter will briefly review cell wall composition and structure, pre-treatment processes, hydrolysis techniques and hydrolytic enzymes.

3.1 Cell wall structure and composition

A large portion of the biomass that will be used for cell wall biorefining will be made up of cell walls that contain polysaccharides and lignin. These cell walls occur in certain cell types, such as fibres, vessels or tracheids. During cell growth, these cells are surrounded only by primary walls, which are mainly composed of polysaccharides and proteins, although phenolic substances can occur (Carpita and McCann 2000). However, when cell growth ceases, these cells synthesise a secondary wall that contains cellulose (40-50%), hemicelluloses (20-30%) and lignin (20-30%). The secondary walls of these cells usually account for more than 95% by weight of the cell wall material (Fengel and Wegener 1989). During secondary wall biosynthesis also the primary wall becomes impregnated with lignin (Bacic *et al.* 1988, Boerjan *et al*. 2003). The secondary walls are rigid and provide structural support. The impregnation of the walls with lignin makes them hydrophobic and more resistant against attack from microorganisms. The composition of cell walls varies depending on the plant species, tissue type, cell type, region within the cell wall and developmental stage of the cell (Table 3). Cell wall composition can also be influenced by genetic variation within a species, growth conditions and age of the plant. This is important, because cell wall composition and structure affect the saccharification process (see Chapter 4).

Lignocellulosic cell walls are strong composite materials. Cellulose is made up of chains of glucose molecules, which are bonded via hydrogen bonds and form microfibrils with crystalline properties. The microfibrils are embedded in a matrix of hemicelluloses and lignin (Figure 2).

Table 3 Composition of biomass feedstock. Percentage values shown are based on dry weight. Table reproduced from Möller *et al.* 2006a.

Feedstock	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Extractives (%)	Ash (%)	Protein (%)
Corn stover	36.4	22.6 (xylose (18), arabinose (3), galactose (1), mannose (0.6))	16.6	7.3	9.7	-
Wheat straw	38.2	24.7 (xylose (21.1), arabinose (2.5), galactose (0.7), mannose (0.3))	23.4	13	10.3	-
Hardwood	43.3	31.8 (xylose (27.8), mannose (1.4))	24.4	-	0.5	-
Softwood	40.4	31.1 (mannose (22.2), xylose (8.9))	28	-	0.5	-
Switchgrass (late cut)	44.9	31.4	12	-	4.6	4.5

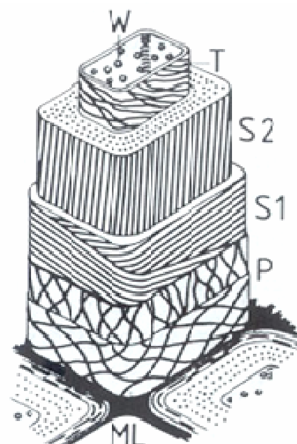

Figure 2 Model of a softwood tracheid cell wall showing the different cell wall layers. The cellulose microfibrils are represented by straight or curved lines, depending on the cell wall layer. ML = middle lamella, P = primary wall, S1 = secondary wall 1, S2 = secondary wall 2, T = tertiary wall (S3), W = warty layer (from Fengel and Wegener 1989).

The hydrophobic lignin molecule impregnates the cell wall, reduces the pore sizes, and shields the polysaccharides (Sell and Zimmermann 1993, Kerr and Goring 1975, Boudet 2003, Saxena and Brown 2005, Ding and Himmel 2006). The hemicelluloses are composed of pentoses and hexoses. They do not have

crystalline properties. The major types of hemicelluloses occurring in lignified secondary walls of dicotyledons are xylans and glucomannans, which can be O-acetylated. Glucuronoarabinoxylan is the major hemicellulose in lignocellulosic material from grasses.

3.2 Pretreatment techniques

For enzymatic saccharification a pre-treatment has to be applied to make the cellulose accessible for hydrolysing enzymes (Mosier *et al*. 2005, Gray *et al.* 2006). At first the raw material can be prepared physically by chipping and washing to reduce feedstock size and remove dirt. Then, biological or chemical pre-treatments are carried out to solubilise parts of the hemicelluloses and the lignin. Several pre-treatment methods can be employed to make cellulose more accessible for the saccharification process. Physical pre-treatment methods are milling and grinding, extrusion and expansion, high-pressure steaming and steam explosion. Chemical pre-treatment methods are alkali treatment, acid treatment, gas treatment, oxidising agents, cellulose solvents, solvent extraction of lignin and swelling agents (Kamm *et al.* 2006b). Biological pre-treatment methods use lignin-degrading organisms, cellulose degrading organisms, or a combination of both (Kamm *et al.* 2006b). The most intensively studied pre-treatment method is steam explosion with and without addition of acid catalyst (Galbe and Zacchi 2002). As shown in Figure 1 the pre-treatment is an integral part of the biorefinery process, when enzymatic saccharification is applied. Depending on the end-product the most suitable pre-treatment process has to be selected. A complete removal of the hemicelluloses from the raw material might for instance not be desired when also the pentoses should be fermented in the following processing steps.

3.3 Hydrolysis techniques

Lignocellulosic materials are composed of more than 70% carbohydrates, which are made up of different monosaccharides. These monosaccharides can be released through acid- or enzyme-catalysed hydrolysis (saccharification) and used as raw

material for new products (US Department of Energy 2004, Lichtenthaler 2006). The conversion of sugar polymers to their monosaccharides at first seems to be straightforward, as only the hydrolysis of glycosidic bonds is required. This is true for the non-cellulosic polysaccharides, which can be degraded by a mild acid treatment at elevated temperatures. However, cellulose is present in a crystalline form, which inhibits access of acid to the interior parts of the cellulose microfibrils.

3.3.1 Acid hydrolysis

In the past various acid hydrolysis technologies have been developed. They are principally based on two processes that were developed prior to and during the time of World War II. These are the Bergius-Rheinau and the Scholler-Tornesch processes. The different acid hydrolysis technologies can be divided into two broad categories: i) hydrolysis with concentrated acid at low temperatures; ii) hydrolysis with dilute acid at high temperatures (Fengel and Wegener 1989).

An example of a concentrated acid hydrolysis process is the Bergius-Rheinau process that uses concentrated, cold hydrochloric acid. Initially, the hemicelluloses are removed from the lignocellulose during a pre-hydrolysis step with 35% hydrochloric acid. Subsequently, the main hydrolysis with a highly concentrated hydrochloric acid is carried out. After this main hydrolysis step, diluted acid is used to treat the hydrolysate. Pre- and main-hydrolysis steps are carried out at low temperatures, and the subsequent treatment of the hydrolysate is carried out at high temperatures and low acid concentration. The acids are recovered by an azeotropic distillation at different pressures, which yields concentrated acid (Fengel and Wegener 1989).

Hydrolysis with highly concentrated acids at low temperatures ensures a slow degradation of the sugar polymers. Sugar recoveries of 90% can be achieved. Problems associated with processes involving concentrated acids are equipment corrosion and energy demand for acid recovery (Galbe and Zacchi 2002). The company Arkenol (Arkenol 2006) has built a technology centre including a pilot plant

in Orange, California, based on the concentrated acid process and this technology is also being used in the Izumi pilot plant in Japan (Bluefire Ethanol 2006).

An example of the dilute acid hydrolysis is the Scholler Tornesch process. Diluted sulphuric acid is used at high temperature and pressure. The high temperature leads to a fast degradation of the polysaccharides, but also to a fast degradation of the released monosaccharides. To limit the degradation of the monosaccharides, hydrolysis is carried out in batches in percolation reactors and the released monosaccharides are quickly removed from the reaction zones. Hydrolysis is carried out with 10-20 subsequential hydrolysis steps. Then, the temperature is raised and the concentration of the acid is reduced. Process modifications have reduced the reaction time to 3-4 h (Madison Scholler Process) (Katzen and Schell 2006).

3.3.2 Enzymatic hydrolysis

A major disadvantage of acid hydrolysis is the potential degradation of the released monosaccharides that leads to reduced sugar yields. Enzymatic degradation of lignocellulosic biomass is very specific and side reactions such as degeneration of sugars do not occur. High yields are therefore possible. In addition, the mild conversion conditions lower maintenance costs of the production plant. Although acid hydrolysis methods are more mature, many experts see the enzymatic hydrolysis of lignocellulosic as a key to cost-effective production of monosaccharides (Hamelinck *et al.* 2005, EERE 2006, US Department of Energy 2006a). An effective pretreatment, which increases the accessibility of the enzymes to the substrate, is necessary for enzymatic saccharification with current enzyme mixtures (US Department of Energy 2006a). Enzymatic cellulose hydrolysis, however, is still an inefficient process. For example, hydrolysis of pre-treated biomass requires 100-fold more enzyme than hydrolysis of starch and is therefore costly (Tolan 2006). Furthermore, cellulases are still costly, although the US DOE National Renewable Energy Laboratory (NREL), together with the enzyme producers Genencor International and Novozymes Biotech, have reduced the enzyme cost 20-30 fold in 2004 (NREL 2004). The costs have to be reduced further.

For example, amylase preparations which hydrolyse starch cost about 1 to 2 US cents per gallon ethanol produced, whereas cellulase preparations still cost about 10 US cents per gallon ethanol (US Department of Energy 2006a).

3.4 Enzymes for the hydrolysis of cell walls

Lignocellulosic material can be degraded and digested by various microorganisms using lignocellulolytic enzymes. For a complete degradation of the material several hydrolytic enzymes are necessary and often communities of microorganisms act together. In principle, the lignocellulolytic enzymes can be categorized into several groups according to the substrate they are degrading: i) cellulases; ii) hemicellulases; and iii) ligninases (oxidases, peroxidases and laccases that act on lignin) (Coombs 1996). Within these groups enzymes differ from each other, for example in their catalytic properties or their substrate specificity (Coombs 1996).

The cellulases, for example, are the cellulose-degrading enzymes. Because of their importance for the saccharification process, they have traditionally received much attention, and research for optimizing cellulose activity and to reduce enzyme cost has been intense. A cellulase system is made up of several enzymes that form a complex. The endoglucanases act on the cellulose microfibrils releasing glucose chains from its surface. The cellobiohydrolases then hydrolyse the 1,4 *β* D-linkages of the free cellulose chains and release cellobiose, which is a glucose dimer. The *β*-glucosidases finally hydrolyse the cellobiose to glucose molecules (Coombs 1996). The cellulase system of *Trichoderma reesei* for example consists of at least five endoglucanases, two cellobiohydrolases and at least two *β*-glucosidases. In addition hemicellulases have been identified that represent only a small portion of the total cellulase system (Zhang and Lynd 2004, Teter *et al.* 2006). The effectiveness of the cellulases in hydrolyzing the substrate is influenced by factors such as product inhibition, thermal inactivation and irreversible adsorption of the enzymes to the substrate (Mansfield *et al*. 1999). Cellulases often have two domain structures, a catalytic domain (CD) and a cellulose-binding domain (CBD). Adsorption of the enzymes to the surface is facilitated by the CBD. Through which mechanism the

CBD attaches to the cellulose microfibril has not been elucidated in detail. Two different theories have been proposed: one states that the CBD serves to increase the local concentration of enzyme at the substrate and the other states that the CBD reacts on the cellulose microfibril and releases cellulose chains from the surface by a non-hydrolytic mechanism. Removal of the CBD has been shown to decrease the hydrolytic efficiency and it has also been shown that addition of a CBD to an enzyme results in increased activity (Mansfield *et al.* 1999). For a comprehensive review about microbial cellulase enzyme systems the reader is referred to the review by Lynd and co-workers (Lynd *et al.* 2002).

The hemicellulases degrade the different hemicelluloses in lignocellulosic material. Examples are xylanases and galacto-glucomannan active mannanases (Teter *et al.* 2006). It has been found that they act synergistically with cellulases, which makes the saccharification process more efficient (Berlin *et al*. 2005).

Lignin is degraded by the action of various oxidases, peroxidases and laccases (Martinez *et al.* 2005). The enzymes catalyse the formation of free radicals, which leads to more cleavage reactions (enzymatic combustion) (Teeri 2004). So far, several studies have been conducted to assess the effect of pre-treating biomass with white rot fungi that secret these ligninolytic enzymes (Itoh *et al*. 2003, Taniguchi *et al*. 2005).

4 CELL WALL CHARACTERISTICS INFLUENCING ENZYMATIC SACCHARIFICATION

Enzymatic saccharification of biomass is influenced by the structure of the feedstock, by cell wall characteristics of the feedstock, by the hydrolysis conditions, including enzyme mixture, and by the pre-treatment used (Mansfield *et al.* 1999, Converse 1993, Chiang and Holtzapple 2000).

The structure of the biomass can influence the penetration of the material with the enzyme solution used to saccharify the cell walls. Different structural levels are important: the tissue level, the cell level and the cell wall level.

Cell wall characteristics that have been considered to affect hydrolysis rate are:

- Particle size/specific surface area
- Cellulose crystallinity
- Cellulose reactivity
- Degree of polymerisation
- Lignin content
- Degree of O-acetylation

4.1 Particle size/specific surface area

Enzymatic saccharification depends on the contact of the enzymes with the substrate. If a given sample is broken down into particles, fractions with small particles have a higher surface to weight ratio. Theoretically it should follow that more hydrolytic enzymes can adsorb to the substrate, which would lead to an increased saccharification rate. However, conflicting results have been reported on the influence of particle size on saccharification efficiency. In a study using pre-treated wood as substrate it was indeed found that saccharification rate increased with decreased particle size. However, pre-treatment not only reduced the size of the particles, but also changed other characteristics of the substrate (Sawada *et al.* 1987). These changes might have led to a better digestibility of the substrate. In a

study using microcrystalline cellulose and cotton linters no correlation between particle size and saccharification were found (Sinitsyn *et al.* 1991). Sangseethong and co-workers however found that particle size influenced saccharification rate (Sangseethong *et al.* 1998) and Gharpuray and co-workers stated that the specific surface area, followed by lignin concentration and cellulose crystallinity were the most important cell wall properties influencing cell wall saccharification (Gharpuray *et al*. 1983).

4.2 Cellulose crystallinity

Cellulose crystallinity has often been inferred to affect saccharification rate strongly (Mansfield *et al.* 1999, Converse 1993). It seems logical that the amorphous regions of the cellulose are hydrolysed first, and that the crystalline regions are degraded poorly (Mansfield *et al* 1999). It was reported that an increase in cellulose crystallinity is accompanied with a decreased hydrolysis rate (Sangseethong *et al.* 1998). Negative correlations of cellulose crystallinity with saccharification efficiency were reported for pure cellulose substrates (Sinitsyn *et al.* 1991). However, other studies reported that cellulose crystallinity did not affect cell wall saccharification (Mansfield *et al.* 1999, Converse 1993). Puri found that saccharification efficiency was not influenced by cellulose crystallinity in pre-treated lignocellulosic biomass (Puri 1984). A similar result was obtained by Sinitsyn and co-workers for a lignocellulosic substrate (Sinitsyn *et al.* 1991) and by Kim and Holtzapple for pre-treated corn stover (Kim and Holtzapple 2006). Cellulose structure is still not understood in detail and therefore it is not known which microstructures influence the activity of cellulases.

4.3 Cellulose reactivity

It was often observed that hydrolysis rate declined with time. Yang and co-workers studied the sugar conversion rate of enzymes using Avicel cellulose as a substrate (Yang *et al.* 2006a). They found, as was observed previously, that the rate of cellulose hydrolysis declined rapidly over time. What was the reason for this? Did

the substrate change during hydrolysis, were the cellulases inactivated or inhibited by the products, or was the substrate surface blocked with the enzymes? Yang and co-workers "cleaned" the surfaces of the cellulose after different reaction times by degrading the enzymes using proteinase. After restart of hydrolysis by adding new enzymes, high hydrolysis rates were again observed. This led to the conclusion that substrate reactivity does not change during hydrolysis, but that the surface of the substrate is blocked by the hydrolytic enzymes.

4.4 Degree of polymerisation

The degree of polymerisation is defined as the number of glucosyl residues per cellulose chain (Mansfield *et al.* 1999). Therefore, a material with a lower DP has more cellulose chain ends. The DP varies between different substrates and is also affected by the preparation of the material. As described above, endoglucanases act on the cellulose chains of the microfibrils and release chain ends. This activity seems not to be influenced by the DP (Zhang and Lynd 2004). Cellobiohydrolases exhibit a higher activity on substrates with low DP. Sinitsyn and co-workers studied the correlation between DP and hydrolysis rate of a number of different substrates and found that DP did not affect hydrolysis rate (Sinitsyn *et al.* 1991).

4.5 Lignin content

Lignin content of lignocellulosic material is negatively correlated with saccharification efficiency (Converse 1993, Mansfield *et al.* 1999, Chiang and Holtzapple 2000). The lignin protects the sugar polymers from enzymatic attack. Separation of lignin from cellulose during pre-treatment is therefore important for increasing saccharification efficiency (Cowling and Kirk 1976). The lignin does not have to be completely removed to enhance digestibility. Tanahashi and co-workers found that it agglomerated into small particles that were attached to the cell walls (Tanahashi *et al.* 1983). Donaldson and colleagues also detected agglomerated lignin particles on cell walls of pine wood after pre-treatment by steam explosion (Donaldson *et al.* 1988). They treated the wood with sodium hydroxid and smeared

out the lignin particles. The lignin now shielded the cellulose microfibrils and hydrolysis rate was greatly reduced (Wong *et al.* 1988, Grabber 2005). How lignin structure, composition and cross-linking affect enzymatic saccharification is not understood in detail (Grabber 2005). It was also found that hydrolytic enzymes are adsorbed on lignin in lignocellulosic substrates (Yang and Wyman 2006b).

4.6 Degree of O-acetylation

The degree of O-acetylation has been shown in several studies to affect saccharification (Converse 1993). It is thought that the acetyl groups sterically hinder the hydrolysing enzymes (US Department of Energy 2006a).

4.7 Conclusion

A better understanding of the cell wall characteristics affecting saccharification is needed. This understanding will contribute to the identification of feedstock with improved cell wall characteristics for biorefining and is essential for designing tailor-made cell walls. Of special importance is the characterisation of cellulose, because of its recalcitrance to enzymatic hydrolysis. Ideally its structure should be studied *in muro* to avoid structural changes caused during cellulose isolation.

5 UNDERSTANDING CELL WALLS: NOVEL ASSAYS FOR CHARACTERISATION AND DIGESTIBILITY

5.1 Biomass potential and cell wall diversity

Different types of feedstock could be used for cell wall biorefineries. Examples are perennial grasses, trees and crop residues. The natural variability of these plants is vast. Furthermore, they can be harvested and used at different stages of maturity, which also influences cell wall composition and digestibility. To identify the most suitable feedstock for biorefineries the cell wall properties must be assessed and correlated with their digestibility. To enable a quick assessment of cell wall characteristics of numerous samples, from different plant species/varieties and from different developmental stages of maturity, high throughput (HTP) assays are needed. This need was identified during a workshop on biofuels and biomass organised by the US Department of Energy (US Department of Energy 2006a) and during the first EPOBIO workshop (EPOBIO report 2006). HTP assays for cell wall characterisation and digestibility will help in identifying varieties of non-food crops with cell walls that are easier to break up. So far, no major breeding efforts have been made to produce crop species with optimised cell walls for saccharification. Crop plants grown for the purpose of food supply on the other hand have been selected and bred for thousands of years, and impressive improvements in yield were accomplished (European Plant Science Organisation EPSO 2005, USDA-NASS 2006) (Figure 3). These gains should also be possible for breeding of dedicated energy crops with desired characteristics, for example high biomass yield and better degradability of the cell walls. An example for the potential of using the natural species variability in relation to pulping efficiency was given by Stewart and colleagues. They analysed the influence of lignin content and structure of different poplar clones on pulping efficiency and concluded that the great variability in the natural resource and breeding of specific clones will enable more cost-effective pulping in future (Stewart *et al.* 2006). Similarly, the identification of plant varieties with better digestibility of their cell walls can help in developing cost-efficient saccharification processes. Targeted breeding, however, depends on knowledge

about cell wall characteristics and cell wall degradability of the plant species (Dinus *et al.* 2001). This chapter will introduce analytical methods that can be used to characterise the chemical composition of cell walls and cell wall degradation products formed in digestibility assays, and methods used to assay cell wall digestibility.

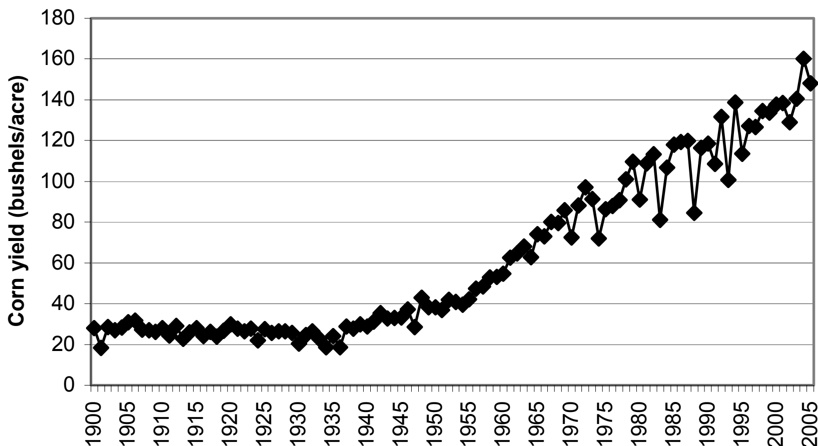

Figure 3 US corn yields 1990-2005. The increased yields are a result of plant breeding, and also of improved agricultural techniques. 39.4 bushel corn = 1 mt. Based on USDA-NASS (USDA-NASS 2006).

5.2 Analytical methods

5.2.1 Classical analytical techniques

A number of different methods are available to characterise cell walls. Traditional “wet chemistry methods” are often labour-intensive, low-throughput and require a considerable amount of cell wall material (Lerouxel *et al.* 2002, Immerzeel and Pauly 2006). Portions of specific cell wall polymers are often extracted and further degraded. For example the analysis of monosaccharides involves acid hydrolysis, potential derivatisation, and analysis by high performance liquid chromatography

(HPLC) or gas chromatography – mass spectrometry (GC-MS) (Immerzeel and Pauly 2006). Lignin analysis by thioacidolysis or derivatisation followed by reductive cleavage (DFRC) also requires solubilisation, derivatisation and analysis by GC-MS (Lu and Ralph 1997). Other traditional methods that are suitable for HTP analyses are colorimetric assays for quantification of sugars (Blumenkrantz and Asboe-Hansen 1973, Dische 1962). These methods could give a quick analysis of the hydrolysis output, however, they are not specific in terms of monosaccharide composition.

5.2.2 Spectroscopy techniques

In the food processing industry HTP quality control is often done using near infrared spectroscopy (NIR), which is a fast analysis technique (Osborne 2000). NIR is also a useful analysis tool for cell wall composition of different plant species (Shenk *et al.* 1992, Poke *et al*. 2004, Schimleck *et al.* 2004, Yeh *et al.* 2004). In addition to its low cost, the technique requires only a simple sample preparation and a small amount of material (Yamada *et al*. 2006). In a study describing the analysis of wood meal pellets of transgenic aspen trees using transmittance NIR spectroscopy, it was shown that correlations could be obtained between NIR data and chemical composition determined by wet chemistry. Correlations were obtained for lignin content, syringyl to guaiacyl ratio, cellulose content and xylose content (Yamada *et al.* 2006). NIR was also used to analyse cell wall characteristics of mature stems from *Arbabidopsis* (Barriere *et al.* 2006). Characteristics analysed were neutral detergent fibre (NDF), acid detergent lignin (ADL), pectin content, soluble carbohydrates, nitrogen content and enzymatic solubility. In maize stems acid detergent fibre (ADF), *p*-coumaric acid and ferulic acid content have also been estimated (Argilliere *et al.* 1995). Like all spectrometric techniques, NIR requires statistical methods, such as multivariate analysis, for data interpretation and correlation with cell wall properties. The spectra are fingerprints composed of overlapping resonances from different cell wall components. To distinguish those fingerprints and relate them to specific cell wall properties a large sample set has to be analysed. Then the calibration set has to be tested against another large sample

group. To set up the calibration, the chemical composition of all these samples has to be determined using traditional wet chemistry methods. Once this calibration step is achieved, 100-1000 samples can be measured per day (Kelley *et al.* 2004). It is important to mention that this calibration is only valid for the selected assay conditions. If plant material or cell wall preparation methods change, a new calibration matrix has to be established.

Fourier Transform Infrared Spectroscopy (FTIR) also allows fast fingerprinting of cell wall composition. In combination with statistical analyses such as principal component analysis (PCA) it is possible to distinguish between cell wall materials from different plant plants (McCann *et al.* 1992, Kemsley *et al.* 1995). The method has been used to identify plant species and *Arabidopsis* mutants (Chen *et al.* 1998, Mouille *et al.* 2003). In contrast to NIR, the FTIR spectra have a higher resolution and specific resonance bands can easier be assigned to cell wall components (Sene *et al.* 1994). FTIR can be used for example to determine lignin content, cellulose content and cellulose crystallinity (ester content, free carboxylgroups). Advantages of FTIR are that only small amounts of samples are needed and that the analysis is fast. Both, NIR and FTIR have the advantage that the samples are not destroyed during analysis and can be used for further analysis by other methods.

Nuclear magnetic resonance (NMR) spectroscopy is a powerful technique that not only provides fingerprints, but also detailed information about cell wall components (Ralph *et al.* 2001, Booten *et al.* 2003). Solid state NMR can be used to quantify, for example lignin and cellulose contents. The technique is non-destructive, but the amount of sample needed is substantial. Depending on the set-up, acquisition of the spectra can take several hours. Also sensitivity and resolution of the technique are lower, compared to solution state NMR. An HTP platform for analysing cell walls by solid state NMR has not been developed, and solid state NMR might be more useful as a benchmarking method for calibrating or validating other techniques. Solution state NMR has been successfully used to characterise solubilised lignin of cell walls (Ralph *et al.* 1999). Recently, a novel cell wall solubilisation method was developed

that allows the fingerprinting and structural analysis of the whole cell wall by solution state NMR (Lu and Ralph 2003, Ralph and Lu 2004). So far it has been shown that this method is applicable to characterise the lignin in cell walls (Lu and Ralph 2003, Ralph and Lu 2004). Whether this method will also be applicable for characterising the carbohydrate components of the wall remains to be shown. A disadvantage of this technique is the low throughput. Also, data analysis is involved and statistical methods for automated analysis of the spectra and prediction of cell wall properties have not been developed.

Other spectroscopic methods that have not been used for HTP analysis are Raman microscopy, UV microspectrophotometry and X-ray diffraction. Raman microscopy and UV microspectrophotometry (UMSP) can also be applied for topochemical investigations. Raman spectroscopy provides information on molecular vibrations that complement infrared spectroscopy, since both methods work with different principles (Gierlinger and Schwanninger 2006). Raman spectra can be acquired on unembedded and untreated sections and allow to discern even thin cell wall layers (Gierlinger and Schwanninger 2006). The spectra are rather complex with overlapping bands and especially hemicelluloses and cellulose are difficult to discern. In the study of Gierlinger and Schwanninger the skeletal vibrations of the hemicelluloses resulted in broad bands and it was not possible to draw conclusions regarding the distribution of the hemicelluloses in the cell walls. UV microspectrophotometry (UMSP) is a good method to analyse the lignin topochemistry of a sample (Koch and Kleist 2001, Möller *et al.* 2006b). The lignin concentration within a certain cell wall area can be estimated, as well as the lignin composition. Sample preparation for this technique involves embedding in a resin and cutting using an ultramicrotome. This analysis cannot be automated because the cell wall areas of interest have to be selected by an operator. Nonetheless, this method might be very useful to analyse lignin distribution of selected samples. Another useful method that has not been applied in HTP is X-ray diffraction. This technique has been described in 1961 as a method to determine crystallinity in polymers (Ruland 1961). However, the determination of cellulose crystallinity in lignocellulosic material is difficult because of its complex structure and composition

and the small size of the crystallites (Andersson *et al.* 2003, Thygesen *et al.* 2005). X-ray diffraction will be an excellent method for calibrating other HTP methods for cellulose crystallinity determination and there could be scope to increase the throughput of this method.

5.2.3 Pyrolysis techniques

Pyrolysis is a destructive method that involves the rapid heating of a sample under the exclusion of oxygen (Galletti and Bocchini 1995). The sample is broken down into its building blocks and the volatile degradation products are analysed using various detectors. Pyrolysis GC-MS is a widely applied technique. The degradation products are separated by gas chromatography and provide a fingerprint of the analysed sample. The breakdown products are further analysed and identified by MS (Ralph and Hatfield 1991). This analysis can, depending on the selected GC conditions, take up to 1h. Other pyrolysis techniques omit the GC step and the breakdown products are immediately analysed by infrared spectroscopy or MS. Pyrolysis MS allows sample analysis within minutes (Labbe *et al.* 2005). A disadvantage of pyrolysis is that linkages within the cell wall cannot be analysed. This information is lost during breakdown of the cell wall. Monosaccharide composition and lignin content can be determined. Only a relatively small amount of sample is needed (1 -10 mg). With an autosampler interface the US National Renewable Energy Laboratory (NREL) was able to screen about 400 samples per day. In combination with multivariate statistical data analysis Pyrolysis molecular beam MS is a very powerful and sensitive technique. NREL is planning on analysing close to 10,000 samples this year.

5.2.4 Enzymatic oligosaccharide profiling techniques

Selected polysaccharides have often been characterised using classical cell wall analysis techniques. As mentioned above, these are labour intensive, slow and expensive. Enzymatic oligosaccharide profiling techniques offer another way to generate breakdown products that can be analysed using advanced analysis

methods. The oligosaccharide profiling technique is based on the use of specific enzymes that cleave the polysaccharide of interest. The released oligosaccharides can then be analysed by chromatographic and spectrometric techniques or by electrophoresis (Immerzeel and Pauly 2006). For example, high-performance anion exchange chromatography (HPAEC) has been used to analyse released xyloglucan fragments of *Arabidopsis* mutants. The mutants could easily be identified and the method was found to be highly reproductive (Immerzeel and Pauly 2006). A disadvantage is that the analysis of a single sample can take up to 2 h (Immerzeel and Pauly 2006). Oigosaccharides can be analysed much quicker in combination with MS. An example is matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS). The cleaved oligosaccharides can be spotted onto arrays and samples can be analysed in HTP using an automated system (Immerzeel and Pauly 2006). Each sample can be analysed within a minute and only about 1 µg of sample is needed (Immerzeel and Pauly 2006). Disadvantages of the technique are that monosaccharides cannot be detected and that only semiquantitative data on the relative abundance of the oligosaccharides can be obtained (Immerzeel and Pauly 2006).

5.2.5 Capillary electrophoresis

Capillary electrophoresis (CE) can be used to analyse fluorescently labelled mono- and oligosaccharides from lignocellulosic material. This method can be performed with an array system enabling fast analysis of multiple samples (Khandurina *et al.* 2004a). Recently, Khandurina and colleagues have applied single column CE to analyse acid and enzymatic hydrolysates from lignocellulosic substrates (Khandurina *et al.* 2004b). They were able to identify monosaccharides by spiking with monosaccharide standards. CE has a high separation efficiency. Other advantages are that only small amounts of material are needed and that multiple samples can be analysed in parallel in a short time (Immerzeel and Pauly 2006). CE can provide a fingerprint and known compounds can be identified by their retention time. However, to identify unknown oligosaccharides, CE has to be coupled with MS (Li *et al.* 1998).

Table 4 Overview of different analytical methods and their features.

* The analysis times stated refer to the machine time, additional time for sample preparation is needed for most methods; also the time needed for data analysis is not included. HTP = high throughput.

Method	Preparation required	Sample amount	Analysis time*	HTP method	Data obtained
Near infrared spectroscopy (NIR)	None or grinding	100 mg	2 min per sample	Established	Spectra have to be analysed in combination with statistical methods
Fourier transform infrared spectroscopy (FTIR)	None or grinding	2-20 mg	2 min per sample	Established	Certain peaks can be identified. Spectra have to be analysed in combination with statistical methods.
Pyrolysis MS	Grinding	1-10 mg	2 min per sample	Established for Pyrolysis MBMS in combination with autosampler	Peaks can be identified by MS
Pyrolysis GCMS	Grinding	1-10 mg	Up to 60 min per sample	With autosampler possible	GC provides additional fingerprint, peak analysis by MS, quantification is difficult.
High performance anion exchange chromatography (HPAEC)	Grinding, solubilisation	5-15 ug	40-120 min per sample	Single sample	Peaks have to be identified with standards. The method is quantitative and structural isomers can be detected.
Oligosaccharide mass profiling (OLIMP)	Grinding, solubilisation	1 ug	2 min per sample	Multiple samples on an array	Masses of oligosaccharides, not quantitative (only relative abundance).

Table 4 Overview of different analytical methods and their features.

* The analysis times stated refer to the machine time, additional time for sample preparation is needed for most methods; also the time needed for data analysis is not included. HTP = high throughput.

Method	Preparation required	Sample amount	Analysis time*	HTP method	Data obtained
Polysaccharide analysis using carbohydrate gel electrophoresis (PACE)	Grinding, solubilisation, derivatisation with a fluorophore	500 fmol monosaccharides 100 fmol oligosaccharides	45 min per sample	Multiple samples	Monosaccharide and oligosaccharide analysis possible. Gel bands have to be identified with standards or after isolation of the fragment from the gel and MS. The method is quantitative and structural isomers can be identified
High performance capillary electrophoresis (HPCE)	Grinding, derivatisation to supply charge and chromophore	attomol	5-20 min per sample	Multiple samples	Peaks have to be identified with standards or MS. The method is quantitative.
Glycoarrays Comprehensive Microarray Polymer Profiling (CoMPP)	Grinding, solubilisation (eg. use of specific enzymes)	5-20 mg, detection limit depending on antibody used	100 samples in three days	Multiple samples on an array	Quantification of oligosaccharides, monosaccharide analysis not possible
Solid state NMR	Grinding	100 mg	Several hours	Single sample	Relatively low resolution, quantification of several components possible, e.g. lignin content

Table 4 Overview of different analytical methods and their features.

* The analysis times stated refer to the machine time, additional time for sample preparation is needed for most methods; also the time needed for data analysis is not included. HTP = high throughput.

Method	**Preparation required**	**Sample amount**	**Analysis time***	**HTP method**	**Data obtained**
Solution state NMR	Grinding, solubilisation and derivatisation of the material	150 mg ml^{-1}	Minutes to hours	Single sample	Spectra that have to be analysed
Gas chromatography mass spectrometry (GCMS)	Grinding, solubilisation, derivatisation	200 ug	40-80 min	Single sample	Identification and quantification of monosaccharides Determination of glycosidic linkages
Colorimetric assays	Grinding, solubilisation	5 ug	60 min	Multiple samples	Amount of total sugars released
X-ray diffraction	Cutting or grinding	Depends on sample and diffraction method used	Minutes to hours	Single sample	X-ray diffraction pattern has to be analysed

5.2.6 Polysaccharide analysis using carbohydrate gel electrophoresis (PACE)

Monosaccharide and polysaccharide composition of cell walls can be analysed by carbohydrate gel electrophoresis (Goubet *et al.* 2002). The sugars and oligosaccharides are derivatised with a fluorophore and separated in a polyacrylamide gel. Oligosaccharides with the same mass but different monosaccharide composition can be separated. Using PACE both structure and quantity of polysaccharides can be studied (Barton *et al.* 2006). This method has recently been applied for enzymatic fingerprinting of *Arabidopsis* pectic polysaccharides (Barton *et al.* 2006). A disadvantage of this method is the need of standards for the identification of oligosaccharides according to their retention time. To identify bands with unknown oligosaccharides these have to be isolated and identified by MS. However, an advantage is that no major expensive lab equipment is needed.

5.2.7 Carbohydrate microarrays (CoMPP)

The carbohydrate array technique can be used to analyse mixtures of carbohydrates, proteoglycans and glycoproteins (Willats *et al.* 2002). In principle two different types of arrays may be distinguished: function arrays and detecting arrays. Willats and colleagues reported the development of a function array. Here carbohydrate solutions are spotted and immobilised on a microarray that was probed with specific antibodies (Willats *et al.* 2002). These are then labelled with secondary antibodies. The array is imaged with a laser array scanner and analysed with imaging software that detects the relative signal from each spot. This way the relative proportions of specific carbohydrates within a cell wall sample can be determined. Instead of monoclonal antibodies, carbohydrate binding modules (CBM) can be used. Detecting arrays are produced by immobilising specific ligands, such as antibodies, on their surface. These are then probed with carbohydrate mixtures. The binding partners are then captured from the mixture. Subsequently the array is probed with antibodies as described above. The main advantages of CoMPP are that the technique is HTP, that it provides information directly about the cell wall

components rather than their chemical signature or spectra, and that it can be applied for cell walls from different plant species. Different cell wall extraction methods have also been tested and the analysis of enzymatically cleaved cell wall components is possible. The main disadvantage is the restricted availability of specific antibodies and CBMs for detection, however new probes can be generated. The carbohydrate array technique has not yet been used to analyse carbohydrates from lignocellulosic substrates, but could be adapted for this purpose. Using CBMs that are specific for crystalline and amorphous regions of cellulose, it might be possible to gain better understanding of the enzymatic catalysed saccharification of lignocellulosic materials.

5.3 Cell wall digestibility assays

As mentioned in Chapter 4 it is necessary to understand the cell wall characteristics that are influencing saccharification in order to select the best suitable feedstock for cell wall biorefineries and to better understand the interaction of hydrolases with the feedstock. It is desirable that a large number of cell wall samples from different species and varieties will be characterised and also tested for their digestibility. Analytical methods that can be used for detailed cell wall characterisation and others that are suitable for high-throughput (HTP) analysis have been introduced above. This Chapter reviews digestibility assays that have been used to assess cell wall degradability and outlines the research need in this area.

The standard assay system to test the hydrolytic activity of cellulases is a filter paper-based test (Wood and Bhat 1988). Filter paper is inexpensive, reproducible and readily available, and therefore often used to assess the effectiveness of enzyme mixtures to saccharify cell wall material (Coward-Kelly *et al.* 2003, Xiao *et al.* 2004). However, it has been reported that the efficiency of enzymes mixtures tested in a paper assay was different to their efficiency in assays using lignocellulosic material as a substrate (Breuil *et al.* 1992, Berlin *et al.* 2005, Kabel *et al.* 2005). Breuil and colleagues found that the amount of *β*-glucosidase in the enzyme assay solution was essential for effective long-term hydrolysis of steam-

treated aspen wood, but not in a model assay using filter paper as substrate (Breuil et al. 1992). Similar observations were made by Kabel and colleagues (Kabel *et al.* 2005). They found that the performance of several different enzyme preparations differed depending on the substrate used.

Berlin and colleagues developed a high throughput assays system that used lignocellulosic material (Berlin *et al.* 2006). The filter paper was replaced by handsheets made from pulp of pre-treated wood of yellow poplar (Liriodendron tulipifera). Small discs were punched out of these sheets, placed in 96-well microtiter plates, treated with hydrolytic enzymes, and the amount of released glucose measured using an enzyme coupled spectrophotometric assay. The results correlated with assays carried out using loose yellow poplar pulp as substrate in Erlenmeyer flasks. This method needs small amount of buffer and enzyme. When the yellow poplar paper sheets were replaced by filter paper sheets, it was found that the enzymes tested were more effective, confirming that digestibility assays to test enzyme efficiency should be carried out on lignocellulosic material (Berlin *et al.* 2006).

Weimer and colleagues used a different approach to assess the digestibility of lignocellulosic feedstock. They used a ruminal inoculum from cows to digest the material and measured the gas production by the microorganism. The gas production per time can be used as a measure for the digestibility of a sample. They also showed that these results correlated with ethanol production in simultaneous saccharification and fermentation (SSF) assays (Weimer *et al.* 2005). The correlation of cell wall digestibility tested with ruminal inoculum to that tested with fungal enzymes has been recognised and is explained with the fact that both groups of microorganisms have evolved under similar selective pressure (Weimer *et al*. 2005). An advantage of the assay is that it does not have to be carried out under aseptic conditions, which makes he analysis of large sample sets easier. The assay can be carried out on untreated and pre-treated material and seems to be very useful for screening cell–wall digestibility of herbaceous biomass.

The digestibility assays can be performed for two reasons: i) to test the digestibility of a feedstock with known enzyme mixtures; ii) to test the effectiveness of novel enzyme mixtures on a given feedstock. In the first case, the digestibility of the feedstock could be tested without or with pre-treatment. A pre-treatment is needed if cellulases are used as hydrolytic enzymes. As mentioned in Chapter 3, pre-treatment of the biomass greatly affects the digestibility of cell walls and without a pre-treatment enzymatic hydrolysis is very ineffective. Small scale pre-treatment technologies have to be developed if current enzyme mixtures have to be analysed for the effectiveness in degrading a selected feedstock. Using a combined pre-treatment and digestibility assay system, matrices of cell wall material, pre-treatment conditions and enzyme mixtures could be tested for their effectiveness. Pre-treatment conditions also have to be adjusted according to the aim of the whole biorefinery process. If also the pentoses released form the hemicelluloses shall be fermented together with the glucose monomers, the pre-treatment has to be milder to avoid solubilisation and separation of the hemicelluloses from the pretreated biomass. Pre-treatments are often carried out at high temperatures and pressures, and scaling these to small samples will be difficult. On the other hand, alternative process integration could lead to integrated saccharification of the pre-treated biomass and the solubilised hemicelluloses. Assessment of digestibility without pre-treatment could for example be performed with enzyme mixtures containing cellulases, hemicellulases and ligninases, or with rumen inoculum.

Pre-treatment and digestibility assays will be affected by the structure of the raw material and the grinding process used. The heterogenous tissue structure of the feedstock will be disrupted and homogenised. Effect of particle size on digestibility has been studied for poplar wood, switchgrass, ryegrass straw, cellulose, newspaper and cardboard, bagasse and corn fibre (Chiang and Holtzapple 2000). However, these tests were carried out with chipped samples. For bench assays particle sizes will have to be reduced and it has not been analysed how this affects the digestibility of cell walls. It is known that milling of cell walls can affect the crystallinity of cellulose.

To test the effectiveness of novel enzyme mixtures for degrading lignocellulosic material the pre-treatment step can possibly be omitted. This would be useful to identify enzyme mixtures that could be used during biomass pre-treatment, if they are resistant to acid, alkali and high temperatures. Effective enzyme mixtures could help improving the pre-treatment process.

As outlined in Chapter 3, cellulases are inhibited by their breakdown products. Depending on the purpose of the assay, the hydrolysis products therefore have to removed to prevent product inhibition. An assay system to test cellulase efficiency in SSF conditions has been developed at NREL, however was not designed for high throughput (Baker *et al.* 1997).

In addition, the chemical analysis of the hydrolysis outputs should be designed to monitor the amount of monosaccharides, but also to monitor other breakdown products for example oligosaccharides. This will lead to a deeper insight into the specificity and effectiveness of the hydrolytic enzymes and of cell wall structure and composition. Techniques that can be used to assess this were identified above.

The flow scheme in Figure 4 shows a possible set up for high throughput digestibility assays. In a first step the lignocellulosic material is broken up to small particles. It is important that a representative portion of the material is prepared. The sample is then pre-treated (optional) and placed into multi-well plates. Enzyme mixtures are added and after incubation, the hydrolysate will be purified and chemical analyses performed.

5.4 Conclusion

As outlined above several methods are suitable for high throughput (HTP) analysis of cell walls and others have the potential to be further developed for HTP analysis (Table 4). All methods have their advantages and disadvantages and comprehensive cell wall analysis will only be possible if a range of methods is applied and the data interpreted and integrated.

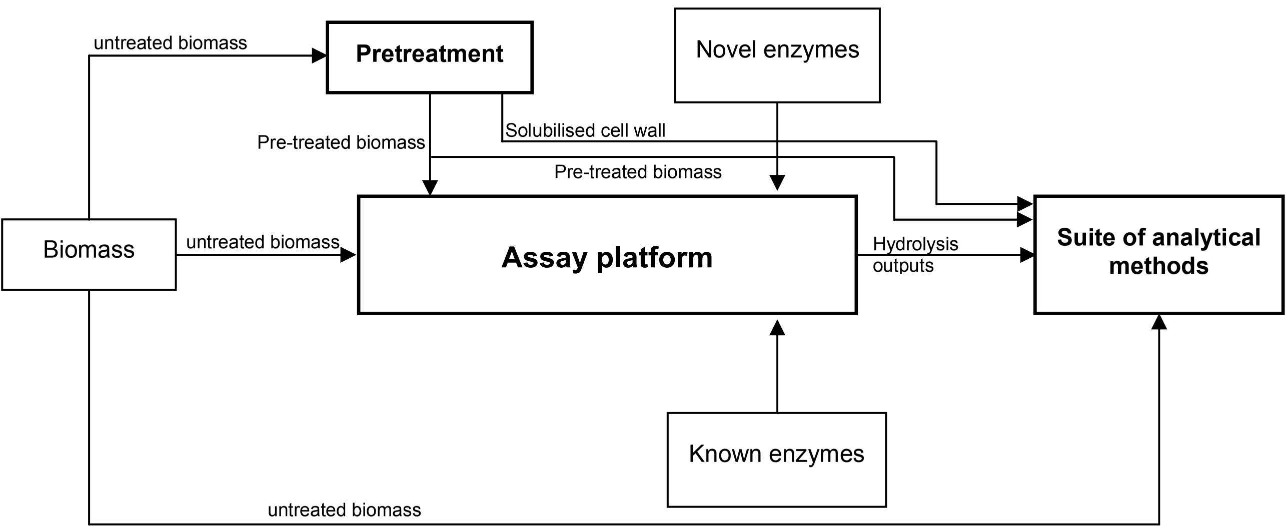

Figure 4 Flow scheme of a high throughput digestibility assay system.

Biomass can be prepared by chipping and grinding, then transferred (and pre-treated) to the multiple sample carrier (assay platform). Untreated and pre-treated biomass samples and solubilised cell wall components in the pretreatment liquid can be analysed using the analytical suite. Novel or known enzyme cocktails are added to the samples on the carrier and incubated. The hydrolysis outputs are then analysed using the analytical suite. The assay conditions have to be adjusted depending on the feedstock used.

Chemometric methods to integrate the data obtained from different methods have to be developed. To achieve this goal, teams of researchers with expertise in applying and developing the different analysis methods have to be formed and work together (see Chapter 7).

Advanced, automated HTP digestibility assay systems with lignocellulosic material are needed for rapid testing of feedstock digestibility and for analysis of the effectiveness of novel hydrolases.

6 IDENTIFICATION OF NOVEL CELLULASES AND OTHER HYDROLASES

As mentioned in Chapter 3, current technologies for cell wall biorefining make use only of a limited range of lignocellulolytic enzymes. A biomass pre-treatment is needed to make the cellulose accessible for the cellulases. In nature, various organisms exist that live on lignocellulosic materials, for example microorganisms that thrive in composts or in rotting wood (Klamer and Bååth 1998). They have evolved very effective enzymatic mechanisms that enable them to degrade the lignocellulosic material without pre-treatment, however, on a rather long timescale. Lignocellulose degrading microorganisms are also found in the digestive tracts of ruminants (Selinger *et al.* 1996) or in termites (Brune 1998, Brune and Friedrich 2000). The genomes of some of these microorganisms have partly been sequenced, for example of the bacteria *Clostridium thermocellum*, *Cytophaga hutchinsonii*, *Microbulbifer degradans*, *Rubobacter xylanophilus*, and of the funghi *Trichoderma reesei* and *Phanerochaete chrysporium* (Martinez *et al.* 2004, Teter *et al.* 2006). The analyses revealed that even simple bacteria had more than 50 genes targeting polysaccharide degradation (Teter *et al.* 2006). Moreover, lignocellulose degrading microorganisms often form communities of thousands of species and it is estimated that less than 1% of these organisms are readily culturable and accessible for biotechnology and basic research (Healy *et al*. 1995, Streit and Schmitz 2004). The life in these communities has led to the evolution of highly specialised and diverse enzyme systems. In general, two distinct cellulase systems have evolved: i) a multicomponent cellulose system of aerobic funghi and bacteria, for example that of *T. reesei*; and ii) the multicomponent cellulosomes of anaerobic bacteria (Teter *et al.* 2006). These facts highlight that these microorganisms will be a rich source for the discovery of novel hydrolases and synergistic enzyme systems that could make technological cell wall saccharification much more effective. The need for designing new enzyme cocktails for saccharification was also pointed out by Berlin and colleagues. As mentioned in Chapter 5 they observed that lignocellulosic material is more resistant to saccharification by cellulases as filter paper, which is almost pure cellulose. They observed that the level of xylanase activity in enzyme mixtures correlated with saccharification efficiency and they

conclude that hemicellulases could make a significant contribution to the effectiveness of enzyme mixtures used for cell wall saccharification (Berlin *et al.* 2005).

Techniques for identifying and characterising novel hydrolases are metagenomics and metaproteomics. Other approaches for enhancing enzyme efficiency are site directed mutagenesis or directed evolution followed by screening for enhanced activity (Berlin *et al.* 2005).

6.1 Metagenomics

So far, less then 1% of all microorganisms have been identified and cultured and the majority of this diversity remains uncharacterised. Metagenomics is a research field that analyses the genomes of whole microorganism communities, the so-called “Metagenome”, without the need for cultivation of selected microorganisms (Streit and Schmitz 2004, Langer *et al.* 2006). In relation to saccharification of lignocellulosics it is important to screen microbial communities that thrive on the lignocellulosic substrate of choice, for example straw or wood. Different strategies are currently exploited to search for novel enzymes. These are homology-driven genome mining, i.e. sequence-based, and activity (function)-based analysis (Ferrer *et al.* 2005a). For homology-driven genome mining the gene library is screened for genes encoding proteins with conserved motifs (*in silico*). Vast amounts of data have to be analysed and novel enzymes might be missed, because these proteins and their sequences are not characterised. For function-based analysis genes are isolated, cloned and expressed in heterologous hosts such as *E. coli*, which can also be combined with a bacteriophage lambda-based expression system (Ferrer *et al.* 2005b). Ferrer and colleagues used this approach and were able to identify novel hydrolases from microbial organisms in bovine rumen. For example, clones can be grown on carboxymethylcellulose and their hydrolytic activity assessed by a simple staining method with Congo red (Healy et al. 1995), or on ostazin brilliant red-hydroxyethyl cellulose and identified by the appearance of a cleared halo zone (Ferrer et al. 2005b). Promising clones can then be expressed in other organisms to

produce large quantities of the enzyme, which can then be tested in digestibility assays for their efficiency in degrading the lignocellulosic material. If this approach is taken, the first screening for hydrolytic activity is obviously very important. Specific screening methods to identify novel hydrolases that might degrade hemicelluloses or lignin still have to be developed.

6.2 Metaproteomics

Metaproteomics offers a new way to learn more about the diversity in microbial communities and to discover novel hydrolases (Wilmes and Bond 2006). Microorganisms living on lignocellulose are secreting hydrolases (secretome). The substrate is extracellularly degraded and the breakdown products taken up by the microorganisms. Targeting of the metaproteomic approach to the secretome therefore leads to a pre-selection of the hydrolases from the housekeeping proteins that are not secreted. Techniques that make it possible to selectively analyse the secretome might therefore be very effective for the discovery of novel hydrolases and have to be developed.

6.3 Conclusion

To identify novel hydrolases by a metagenomics or metaproteomics approach new techniques must be developed. For the function-based analysis of the metagenome, assays with novel substrates, for example lignocellulosic material must be developed. For a metaproteomics approach methods for targeting the secreted hydrolases have to be designed.

7 EPOBIO RECOMMENDATIONS

There is a well recognised need to overcome society's dependency on finite fossil reserves. Major initiatives worldwide are targeting plant-based raw materials as new feedstocks for the manufacture of transport fuels and other biobased products. The development of new biorefineries is occurring globally and is predicted to continue to escalate. Currently, much of this activity is aimed at the production of bioethanol for liquid transport fuels. Increasingly the 'biorefinery platform' will be designed as an integrated process producing a diverse range of products from plant-based raw materials.

The raw material feedstocks can be derived from a number of sources, such as products and co-products from forestry, agriculture, horticulture, aquaculture and waste. Irrespective of the source, raw materials derived from plant biomass are largely composed of cell walls – a polymeric mixture, highly resistant to degradation. For efficient biorefining, the cell walls in the feedstocks must be taken apart in a way that releases their components whilst maintaining the economic value of those components. A key issue is the release of sugars from the cell wall polysaccharides. This presents a major bottleneck for three principal reasons. First, the lignocellulose composition of cell walls represents a significant barrier to accessibility of polysaccharides. Second, cellulases continue to require optimisation for the biorefinery process. Third, novel hydrolases of other cell wall polysaccharides and lignin need to be discovered and combined to optimize bioconversions. Currently, this bottleneck for biomass utilisation is mainly addressed by harsh chemical and physical pre-treatments and process integration. This is expensive in terms of energy input, compromises subsequent bioconversions and potentially leads to loss of economic value of co-products degraded by the harsh conditions.

These issues are strong indicators that alternative approaches must be developed to gain maximum environmental and economic value from biomass used in the biorefinery process. The approaches should involve biobased conversions carried out with minimum energy input and mild conditions that maximize potential utility of

the biomass. An integrated approach combining characterisation of the plant cell wall feedstocks with knowledge of the properties that determine their enzymatic digestibility is essential. This must proceed in parallel to optimisation of hydrolases for cell wall digestion. The EPOBIO study has revealed the strategic need to pursue these two objectives in a linked way to ensure progress is rapid in an area recognized to be so important for the development of the global bioeconomy. In that context, the potential of biorefineries in developing countries should also be examined and taken forward.

7.1 Research and development needs

Targets for understanding cell wall feedstocks and their digestibility

The composition and molecular organisation of plant cell walls is species-, tissue- and cell-specific and is responsive to environmental change. This complexity necessitates the analysis of many different cell wall feedstocks since generic conclusions cannot be drawn from data gained from a single species, organ, developmental stage or crop grown from a single geographical region. It is essential to focus this characterisation on properties that determine enzymatic digestibility. This requires integration of approaches to characterize the cell walls and assays to determine digestibility of those same cell walls. Therefore, R&D should focus on a diverse range of biomass feedstocks, developing molecular and analytical tools for their characterisation in parallel to the design of novel high throughput assays for their digestibility. These assays must be complemented with research into cell wall pre-treatment. It is probable that the scale of work is such that international and multidisciplinary collaboration is required.

Targets for discovery and optimisation of cellulases and other hydrolases

The use of cellulases is fundamental to successful biorefining. R&D should focus on gene discovery and protein engineering approaches to further optimise cellulases. Hydrolases of cell wall polysaccharides are linkage-specific and there is

considerable potential for the discovery of novel hydrolases, particularly from microorganisms. R&D should use metagenomics and metaproteomic strategies to identify novel hydrolases, both of cell wall polysaccharides and of lignin.

Integrating understanding of cell walls, digestibility and hydrolytic actions

Ideally the targets described above should be fully integrated in a single research project to ensure exchange of know-how and materials. This will enable continuous feedback and transfer of information to progress each set of R&D objectives effectively.

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EPOBIO is supported by the European Commission under the Sixth RTD Framework Programme Specific Support Action, Science to Support Policy SSPE-CT-2005-022681
together with the United States Department of Agriculture

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Published in the UK by CPL Press

ISBN 10: 1-872691-04-8 ISBN 13: 978-1-872691-04-6