Sustainable Biotechnology

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Editors

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Sources of Renewable Energy

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Applications of Biotechnology for the Utilization of Renewable Energy Resources

Om V. Singh and Steven P. Harvey

Introduction

Even given the seemingly unlikely near-term resolution of issues involving atmospheric CO_2 levels and their effect on the climate, the adoption of global conservation measures, and the stabilization of fossil fuel prices, it is still a certainty that global oil and gas supplies will be largely depleted in a matter of decades. That much is clear from even a cursory comparison of the independent estimates of the world's oil and natural gas reserves and the respective data on their consumption, as published regularly on the internet by the US Government Energy Information Administration [1]. Nature of course, offers abundant *renewable* resources that can be used to replace fossil fuels but issues of cost, technology readiness levels, and compatibility with existing distribution networks remain. Cellulosic ethanol and biodiesel are the most immediately obvious target fuels, with hydrogen, methane and butanol as other potentially viable products. Other recent reports have covered various aspects of the current state of biofuels technology [2–4]. Here we continue to bridge the technology gap and focus on critical aspects of lignocellulosic biomolecules and the respective mechanisms regulating their bioconversion to liquid fuels and value-added products of industrial significance.

The lignocellulosic structure does not readily yield its component five- and six-carbon sugars so the efficient biological conversion of biomass typically requires a pretreatment step to render the polysaccharide molecules accessible to enzymes. Several thermochemical or biochemical approaches are currently in various stages of development, and have the potential for major impact on the economics of biofuel

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production. In order to derive a stable and cost-effective approach, a greater fundamental understanding is needed of the exact effects of these processes on plant anatomy. These are difficult experiments to conduct and in Chapter 1 "Heat and Mass Transport in Processing of Lignocellulosic Biomass for Fuels and Chemicals", *Viamajala* et al. provide an in-depth report on the effects of heat and mass transport on the efficiency of biomass conversion. Further, *Wu* et al. in Chapter 2 "Biofuels from Lignocellulosic Biomass", give the matter a more detailed consideration by comparing thermochemical and biochemical approaches to the production of biofuel from lignocellulosic biomass.

As compared to gas and oil, relatively greater potential reserves exist for both coal and uranium (probably on the order of a century) but neither is renewable and each is associated with its own environmental conundrum (carbon release and waste storage, respectively). Linus Pauling expressed a particular concern for the destruction of the element uranium, saying "In a thousand or ten thousand years the world may require uranium for a purpose about which we are currently ignorant." [5]. Looking beyond the immediate temporal horizon, we are unavoidably confronted with the need to develop permanently renewable sources of energy.

Earth's most plentiful and renewable energy resources typically include sunlight, wind, geothermal heat, water (rivers, tides and waves), and biomass. All of these are suitable for the generation of electricity but biomass is the current main renewable feedstock for the production of "liquid" fuels - typically ethanol, and biodiesel and possibly to include butanol, hydrogen and methane. These liquid fuels, or energy carriers lie at the heart of the solution to the global energy problem, since they are the materials currently most suitable for use in the transportation sector and for the direct replacement of the immediately endangered fossil resources of oil and gas. *Vasudevan* et al. in Chapter 3 "Environmentally Sustainable Biofuels – The Case for Biodiesel, Biobutanol and Cellulosic Ethanol" provide a detailed discussion of the case for ethanol, butanol and biodiesel. Significantly, a potential technical hurdle confronting the production of biofuels is the efficiency of utilization of hemicellulose-derived sugars. In Chapter 4 "Biotechnological Applications of Hemicellulosic Derived Sugars: State-of-the-Art", *Chandel* et al. examine the challenges associated with the successful utilization of this second most abundant polysaccharide in nature.

Energy-yielding materials are found in various guises, one of which is garbage. Although not always classified as a resource, garbage clearly is renewable (increasingly so, in fact), and processes that convert it into energy are obviously dually beneficial. In Chapter 5 "Tactical Garbage to Energy Refinery (TGER)", *Valdes and Warner* present a hybrid biological/thermochemical system designed for the conversion of military garbage into ethanol and electricity, with clear potential for applications in the civilian sector.

Agricultural waste (e.g. livestock, manure, crop residues, food wastes etc.) is a high impact feedstock with particular utility in the production of biogas. In Chapter 6 "Production of Methane Biogas as Fuel Through Anaerobic Digestion", *Yu and Schanbacher* discuss the anaerobic conversion of biomass to methane. Untreated wastewater also contains biodegradable organics that can

be used to produce hydrogen or methane. In Chapter 7 "Waste to Renewable Energy: A Sustainable and Green Approach Towards Production of Biohydrogen by Acidogenic Fermentation", *Mohan* provides a detailed review of the state of the art with regard to biological hydrogen production using waste and wastewater as substrates with dark fermentation processes.

Many biological processes use mixed cultures operating under non-sterile conditions (e.g. biological hydrogen and methane production, as discussed above). *Watanabe* et al. in Chapter 8 "Bacterial Communities in Various Conditions of the Composting Reactor Revealed by 16S rDNA Clone Analysis and Denaturing Gradient Gel Electrophoresis" demonstrate the utility of 16S rRNA analysis and denaturing gradient gel electrophoresis (DGGE) techniques for tracking microbial communities within a mixed and changing culture. Their work uses a composting process, which offers a typically cost-effective alternative to incineration for the remediation of contaminated soil.

The production of liquid fuel from biomass necessitates the consideration of various issues such as the effects on the food supply, the rainforest, and greenhouse gas production, as well as carbon sustainability certification. Some of these issues may require appropriate regulations and in Chapter 9 "Perspectives on Bioenergy and Biofuels"*, Scott* et al., examine these issues closely.

In addition to its environmental advantages, the use of renewable energy resources offers the potential for stimulation of the economies of the nations where they are produced. The potential products of these renewable materials extend well beyond liquid fuels alone. Owing partly to the enormous volume of their production, fuels are sold for relatively low prices, and the successful implementation of renewable fuels depends, at least initially, on their ability to compete in the marketplace. To this end, it is particularly important to maximize the efficiency of their production in biorefineries where secondary products would be derived from the same feedstock as the fuels. As an example, petroleum refineries have been in operation for over 150 years and now produce lubricants, plastics, solvents, detergents, etc., all from the starting crude oil [6]. Similarly, biomass, in addition to being used for the production of fuels, can be used as a starting material for the production of other value-added products of microbial bioconversion processes such as fermentable sugars, organic acids and enzymes. In Chapter 10 "Perspectives on Chemicals from Renewable Resources", *Scott* et al. describe how, with the aid of biotechnology, Protamylase® generated from starch production, can be used as a medium for the production of a cynophycin polymer, which is a major source of arginine and aspartic acid for the production of many industrially useful compounds including 1,4-butanediamine and succinic acid. In Chapter 11 "Microbial Lactic Acid Production from Renewable Resources", *Li and Cui* describe the production of lactic acid from renewable resources such as starch biomass, cheese whey etc. Lactic acid has recently gained attention due its application to the manufacture of biodegradable polymers. Among other renewable resources, Chapter 12 "Microbial Production of Potent Phenolic-Antioxidants Through Solid State Fermentation", *Martin* et al. describe the role of agroindustrial residues including plant tissues rich in polyphenols for the microbial bioconversion of potent phenolics under solid state

fermentation conditions. Hence, combined with the economy of scale derived from large refineries, secondary products could be key to bridging the price gap between fossil fuels and renewables.

One critical advantage of biofuels is their potential to achieve a reduction in greenhouse gas releases, since the plants from which they are produced derive their carbon from the atmosphere. The overall balance of greenhouse gases however, depends in large measure on the particular feedstocks used and the methods by which they are produced. Corn ethanol for instance, while being potentially carbon neutral, is not likely to achieve an overall reduction in greenhouse gas release due to its requirement for nitrogenous fertilizer and the associated release of nitrous oxide [7]. An interesting approach to the production of biodiesel is the use of algae to synthesize oil from the CO_2 they capture for growth. Algae cultivation offers a potential low-cost alternative to physical methods of carbon sequestration such as pumping liquid CO_2 underground or underwater or chemical methods such as base-mediated capture of CO_2 and subsequent burial of the resulting carbonates. The algae, while using CO_2 as their sole source of carbon for growth, can produce up to 50% of their weight in oil suitable for conversion to biodiesel. Algae are one of the best sources of plentiful biomass on earth; their potential for biosynthesis of astaxanthin, a red carotenoid nutraceutical responsible for the color of salmon flesh, was explored in Chapter 13 "Photoautotrophic Production of Astaxanthin by the Microalga *Haematococcus pluvialis*", *Del Rio* et al.

In a biological system, the biosynthesis of industrially useful compounds has long been recommended. Heparin, a low-molecular weight highly sulfated polysaccharide represents a unique class of natural products, that has long been used as an anticoagulant drug. Due to recent outbreaks of contamination and seizure of heparin manufacturing facilities [8], an efficient bioconversion process of heparin is required. In Chapter 14 "Enzymatic Synthesis of Heparin", *Liu and Liu* describe novel enzymatic approaches for the biosynthesis of heparin sulfate that mimic *E. coli* heparosan.

Discovering new and sustainable resources can help refuel industrial biotechnology. Adverse environmental conditions which normal earth microbiota do not tolerate, offer potential sites to explore specific sets of microorganisms designated as "Extremophiles". The discovery of these microorganisms has enabled the biotechnology industry to innovate unconventional bioproducts i.e. "Extremolytes" [9]. In Chapter 15 "Extremophiles: Sustainable Resource of Natural Compounds-Extremolytes", *Kumar* et al. provide an overview of these extreme habitats. The applications of extremophiles and their products, extremolytes, with their possible implications for human use are also discussed broadly.

This book "Sustainable Biotechnology: Sources of Renewable Energy" is a collection of research reports and reviews elucidating several broad-ranging areas of progress and challenges in the utilization of sustainable resources of renewable energy, especially in biofuels. This book comes just at a time when government and industries are accelerating their efforts in the exploration of alternative energy resources, with expectations of the establishment of long-term sustainable alternatives to petroleum-based liquid fuels. Apart from liquid fuel this book also

emphasizes the use of sustainable resources for value-added products, which may help in revitalizing the biotechnology industry at a broader scale.

We hope readers will find these articles interesting and informative for their research pursuits. It has been our pleasure to put together this book with Springer press. We would like to thank all of the contributing authors for sharing their quality research and ideas with the scientific community through this book.

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Heat and Mass Transport in Processing of Lignocellulosic Biomass for Fuels and Chemicals

Sridhar Viamajala, Bryon S. Donohoe, Stephen R. Decker, Todd B. Vinzant, Michael J. Selig, Michael E. Himmel, and Melvin P. Tucker

Abstract Lignocellulosic biomass, a major feedstock for renewable biofuels and chemicals, is processed by various thermochemical and/or biochemical means. This multi-step processing often involves reactive transformations limited by heat and mass transport. These limitations are dictated by restrictions including (1) plant anatomy, (2) complex ultra-structure and chemical composition of plant cell walls, (3) process engineering requirements or, (4) a combination of these factors. The plant macro- and micro-structural features impose limitations on chemical and enzyme accessibility to carbohydrate containing polymers (cellulose and hemicellulose) which can limit conversion rates and extents. Multiphase systems containing insoluble substrates, soluble catalysts and, in some cases, gaseous steam can pose additional heat and mass transfer restrictions leading to non-uniform reactions. In this chapter, some of these transport challenges relevant to biochemical conversion are discussed in order to underscore the importance of a fundamental understanding of these processes for development of robust and cost-effective routes to fuels and products from lignocellulosic biomass.

Keywords Lignocellulose · Biomass · Biofuels · Heat transport · Mass transport

1 Introduction

The biochemical conversion of lignocellulosic biomass requires several processing steps designed to convert structural carbohydrates, such as cellulose and hemicellulose, to monomeric sugars, which include glucose, xylose, arabinose, and mannose. These sugars can be fermented to ethanol and other products, to varying degrees of effectiveness, by wild type and modified microbial strains. The front end of the process includes feedstock size reduction followed by a thermal chemical treatment, called pretreatment. In practice, this unit operation usually involves the exposure of

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biomass to acid or alkaline catalysts at temperatures ranging from 120 to 200°C. Pretreated slurries (the hydrolysate liquor containing soluble sugars, oligosaccharides, and other released solubles plus the residual solids) are then enzymatically digested at 40–60°C to release sugars from the polysaccharides and oligomers remaining after pretreatment [1–9]. In both of these steps, adequate heat, mass, and momentum transfer is required to achieve uniform reactions and desirable kinetics.

Plant cell walls, which make up almost all of the mass in lignocellulosic biomass, are highly variable both across and within plant tissue types. At the macroscopic scale, such as within a stem or leaf, uneven distribution of catalyst (chemical or enzyme) due to the different properties of different tissues results in heterogeneous treatment, with only a fraction of the plant material exposed to optimal conditions [10–13]. Tissues that do not get exposed to sufficient amounts of catalyst during pretreatment are incompletely processed, resulting in decreased overall enzymatic digestibility of pretreated biomass [6]. When pretreatment severity is increased, by increasing temperature, catalyst concentration, or time of reaction, areas of biomass readily exposed to catalyst undergo excessive treatment leading to sugar degradation and formation of toxic by-products (furfural, hydroxymethyl furfural, and levulinic acid) that inhibit downstream sugar fermentation and decrease conversion yields [1]. This problem continues at a microscopic scale due to the compositional and structural differences between middle lamella, primary cell wall, and secondary cell wall. At even smaller scales, intermeshed polymers of cellulose, hemicellulose, lignin, and other polysaccharides present another layer of heterogeneity that must be addressed during bioconversion of plant cell walls to sugars.

Milling to fine particle sizes improves some of these mass transfer limitations, but can add significant costs [14, 15]. Size reduction, however, may not overcome heat transfer limitations associated with short time-scale pretreatments that employ hot water/steam and/or dilute acids. When such pretreatments are carried out at high solids loading (>30% w/w) to improve process efficiency and increase product concentrations, heat cannot penetrate quickly and uniformly into these unsaturated and viscous slurries. It is thought that steam added to high-solids pretreatments can condense on particle surfaces impeding convective heat transfer. Depending on particle and slurry properties, the condensed steam can form temperature gradients within biomass aggregates, resulting in non-uniform pretreatment.

Besides limiting heat transfer rates, biomass slurries can pose other processing challenges. At high solids concentrations, slurries become thick, paste-like, and unsaturated. Limited mass transfer within these slurries can cause localized accumulation of sugars during enzymatic hydrolysis, decreasing cellulase and hemicellulase activity through product inhibition [16–23]. In addition, slurry transport through process unit operations is challenging at full scale. As solid concentrations increase, hydrodynamic interactions between particles and the surrounding fluid as well as interactions among particles increase. At high solids concentrations “dense suspensions” are formed and the resulting multiple-body collisional or frictional interactions and entanglement between particles creates a complex slurry rheology [24–26]. A further complicating aspect is water absorption by biomass, causing the bulk to become unsaturated at fairly low insoluble solids concentrations (~ 30–40%

w/w) and behave as a wet granular material [27]. This material is highly compressible and the wet particles easily "stick" to each other and agglomerate. With no free water in the system, the material becomes difficult to shear or uniformly mix.

At the ultrastructural scale of plant cell walls, catalysts must penetrate through the nano-pore structure of the cell wall matrix to access the "buried" and intermeshed carbohydrate polymers. Based on reported average cell wall pore sizes of 5–25 nm [28–31], small chemical catalysts (<1 nm) may not face as significant a penetration barrier as do enzymes (about 10 nm). The most dominant commercial cellulase component enzyme, cellobiohydrolase I or Cel7A, has dimensions of ~5 × 5 × 12 nm [32, 33] which is roughly the same size as smallest of these reported nano-pores, likely restricting accessibility to primarily surface cellulose chains. Once they have penetrated the cell wall matrix, these enzymes must locate suitable substrates. For Cel7A, this implies that a region of cellulose microfibril has been sufficiently unsheathed from lignin and hemicellulose to expose the cellulose core (Fig. 1). This unsheathing process may be accomplished by the pretreatment or as an ablative effect caused by the system of cellulase enzymes which can peel away microfibrils from the surface layers. Lignin is also a major impediment to cellulase action because it is difficult to remove uniformly or modify through pretreatment. Furthermore, it is entirely unclear at this time if lignin can be effectively removed from cell walls using enzymes.

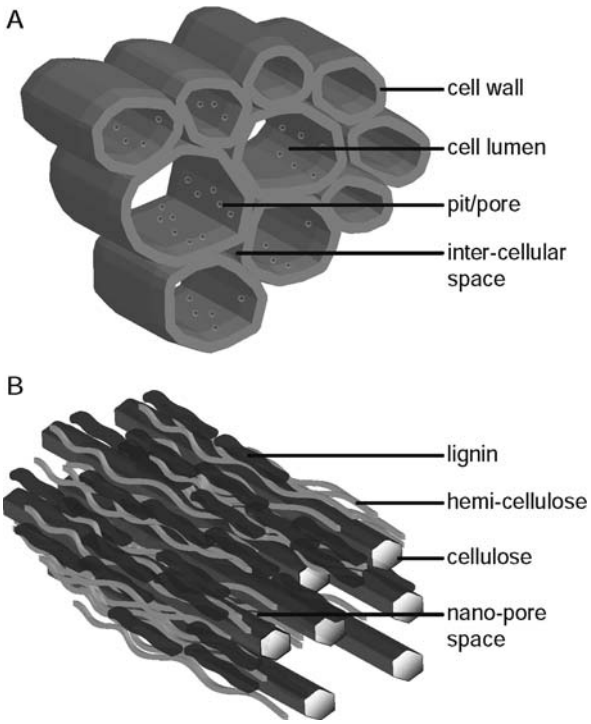

Fig. 1.1 Cartoon depiction of cellular-scale (**a**) and molecular-scale (**b**) obstacles to heat and mass transport in lignocellulosic biomass

Lignin is believed to impede enzymatic hydrolysis of cellulose by interacting with biomass surfaces and either blocking the path of processive hydrolases (e.g. Cel7A), preventing enzymatic access to specific binding sites, or through nonspecific binding of cellulolytic enzymes [34–36] to lignin. Several low-temperature pretreatment protocols, such as alkaline peroxide [37, 38] or lime and oxygen [39], address these issues by removing substantial amounts of lignin. Although these processes are highly relevant to the pulp and paper industry, the fate of lignin and its impact on enzymatic digestibility after high-temperature acidic or neutral pretreatments has largely been neglected until recently [40–42]. Recent observations show that lignin undergoes significant structural changes during high temperature pretreatments. These changes cause it to both mobilize during elevated temperatures and then coalesce upon cooling, both within the cell wall matrix and on the biomass surfaces [40]. This mobilized processed lignin, when redeposited onto cellulose surfaces, can impede enzymatic digestion presumably due to the occlusion of substrate binding sites [42]. All of these transport limitations during lignocellulosic conversion to ethanol impact the overall process performance and thus warrant more detailed further investigation.

2 Macroscopic Transport Through Plant Tissues

In a large-scale process, pre-impregnation of catalyst into large pieces of biomass (>1 cm) is often overlooked; however, milling biomass to reduce this problem can incur large energy and equipment costs [1, 14, 15]. This problem is compounded by the widespread use of process irrelevant biomass sizes for laboratory experiments. Most laboratory studies on biomass to ethanol conversion processes use finely milled materials (20–80 mesh is standard) where the effects of macroscopic transport processes are not easily observed or are masked altogether [43–45]. In larger pilot studies using compression screw feeders, these transport effects can be further masked by the high-shear feeder causing biomass size reduction [6, 8]. Often this size reduction occurs after catalyst impregnation, limiting catalyst effectiveness on pretreatment. A further complication is that compression of the feed stock may cause biomass pore structure collapse, leading to uneven heat and mass transfer during pretreatment [10, 13] as well as limitation of catalyst access to the interior of the biomass.

Before larger biomass particles containing intact tissues are used in processing, it is essential to understand the catalyst transport processes and pathways and the limitations associated with them (Fig. 1). In living plants, vascular tissues such as xylem and phloem are the primary routes for transport of water and nutrients along the length of the plant stem and leaves. Additional transport within tissues and between adjacent cells is carried out through (1) the pits, areas of thin primary cell wall devoid of secondary cell wall between adjacent cells and (2) the apoplast, the contiguous intercellular space exterior to the cell membranes [46]. In dry senesced plants, studies with dyes to visualize fluid movement through tissues showed that the apoplastic space is the major catalyst carrier route, with limited fluid movement

occurring through the vascular tissue [11]. In untreated biomass, the pits do not appear to support significant transport. It is probable that these pits disintegrate and open up during pretreatment allowing fluid to flow through [40]. Thus, new pathways for catalyst penetration are formed either during the drying process that creates fractures in plant tissues or after some degree of biomass degradation.

The primary major barrier to fluid transport into native dry plant tissue appears to be air entrained in the cell lumen. Simple exposure of tissues to high temperature fluids is insufficient to achieve catalyst distribution to all parts of the biomass [11]. The primary escape route for the intracellular air is most likely through pits. However, the small pit openings (approx 20 nm) could be blocked due to cell wall drying and water surface tension may prevent movement through these narrow openings. Forced air removal by vacuum provides additional driving force for the bulk fluid mobility necessary to enhance liquid and catalyst penetration into tissues as demonstrated by Viamajala and coworkers [11]. Heating dry biomass can minimize the amount of entrained air (due to expansion of air by heat) and assist in drawing liquid into the cells by contraction of the entrained air when cooled by immersion in catalyst-carrying liquid. Thus, bulk transport, rather than diffusive penetration, is the dominant mass transfer mechanism into dry biomass.

Although movement of fluids is associated with catalyst transport, the primary goal of catalyst distribution is to deliver the catalyst to cell wall surfaces containing fuel-yielding carbohydrates, rather than to empty cytoplasmic space in dry tissues. In fact, entrainment of fluids in the biomass bulk can be detrimental to small time-scale dilute acid or hot water pretreatments, as the presence of excess water increases the net heat capacity of the material, increasing the heating time needed to achieve desired pretreatment temperatures. Data shown in Fig. 2 support this hypothesis. In this set of experiments, un-milled sections of corn stems

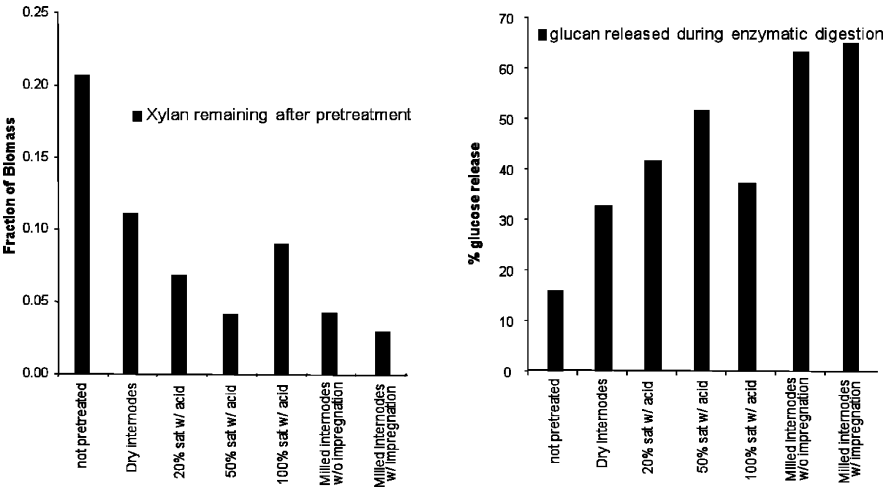

Fig. 1.2 Effect of preimpregnation of corn stover stalks with dilute acid and particle size reduction on (**a**) pretreatment and (**b**) subsequent enzymatic hydrolysis

(~ 1 inch long) were saturated to various degrees with dilute sulfuric acid (2% w/w) and pretreated in 15 mL of the same acid solution at 150°C for 20 min. Milled corn stems (–20 mesh) pretreated under identical conditions served as controls. All pretreatments were performed in 22 mL gold coated Swage-Lok (Cleveland, OH) pipe-reactors, heated in an air-fluidized sand bath [42]. After pretreatment, whole stem sections were air-dried, milled and enzymatically digested for 120 h with a 25 mg/g of cellulose loading of a commercial *T. reesei* cellulase preparation (Spezyme CP, Genencor International, Copenhagen, Denmark) supplemented with an excess loading (90 mg/g of cellulose) of commercial *Aspergillus niger* cellobiase preparation (Novo 188, Novozymes Ltd., Bagsvaerd, Denmark) using procedures described previously [47]. Milled stover pretreated as controls in this experiment was dried and digested similarly, but without any further comminution.

In Fig. 2a, dry internodes pretreated without pre-impregnation of catalyst were poorly pretreated as evidenced by the high amounts of xylan remaining in the biomass after reaction. Stem sections pre-impregnated to achieve 20% saturation showed better reactivity and xylan removal and this trend continued when stem sections pre-impregnated to 50% saturation were pretreated. However, when completely saturated (100%) stem sections were pretreated, xylan conversion was observed to be lower. Milled materials with and without pre-impregnation of catalyst – conditions that would have lowest mass transfer limitations, showed comparable pretreatment performance with each other as well as with the 50% saturated stem sections. These results confirm that only limited catalyst penetration and pretreatment is achieved when air remains entrapped in cytoplasmic spaces such as in dry internodes. Enhanced catalyst distribution and transport dramatically enhances pretreatability up to a certain point, after which excess fluid impedes pretreatment. Similar conclusions on the negative impacts of poor bulk transfer on biomass pretreatability can be inferred from other reported studies also. Tucker and coworkers [10] observed poor pretreatability of biomass during steam explosion of corn stover when materials were not pre-wetted with dilute acid and ascribed their results to mass transport limitations. In another study Kim and coworkers [13] observed poor pretreatment of biomass when the biomass was pressed prior to pretreatment and hypothesized that the mechanical compression of biomass caused pore structure collapse resulting in formation of material that was relatively impervious to heat and mass transfer.

Enzymatic digestion results corresponding to pretreatments shown in Fig. 2a, are presented in Fig. 2b. As expected, release of monomeric sugars from pretreated whole stem sections was proportional to the degree of pretreatment they experienced. Unmilled biomass that was 50% saturated with acid before pretreatment showed better digestibility than the sections that were pre-saturated to lower or higher levels. Milled biomass, however, digested best, demonstrating the importance of enhanced enzyme transport – an outcome of the more thorough and uniform pretreatment of milled materials. With woody feedstocks, milling to fine particle sizes may be impractical and pre-impregnation of biomass with catalyst, as practiced in the pulp and paper industry [48], might need to be utilized to improve conversion efficiencies.

3 Microscopic Transport Through Plant Cell Walls

Enzyme penetration into plant cell wall is widely acknowledged to be a key barrier to economical and effective biochemical conversion of lignocellulosic biomass [5, 49]. In fact, the primary function of pretreatment of lignocellulosic biomass is to assist subsequent enzymatic digestibility by making cell walls more accessible to saccharifying enzymes [1, 4, 44]. However, an accurate description of the methods by which enzymes penetrate cell walls and accomplish cellulose degradation has been lacking. A recent study by Donohoe and coworkers provided, for the first time, direct visual evidence of loosening of plant cell wall structure due to dilute acid pretreatment and the subsequently improved access by cellulases [49]. Figure 3c–f further demonstrate the penetration of cellulases into pretreated cell walls as detected by nano-gold labeled antibodies to Cel7A and other cellulases. This study shows that penetration of enzymes into mildly pretreated cell walls is minimal and that cells stay largely intact even after prolonged exposure to cellulases (Fig. 3a, b). In moderately pretreated cell walls, cellulases are able to partially penetrate and disintegrate the inner secondary layers (S3) only (Fig. 3c, d); whereas the outer layers (S1 and S2) remain impervious to enzymes. In severely pretreated cell walls, enzymes penetrate throughout (Fig. 3e, f). These data suggest that enzymatic digestibility of biomass is restricted by transport of enzymes into cell walls. While not directly evidenced by this study, these results also suggest that thermal pretreatments (and possibly others) "loosen" cell walls in layers providing enzymes access only to these structurally compromised zones of the cell walls. Kinetic data on thermal pretreatments by several research groups also suggests likely mass transfer limited xylan removal that can be modeled as parallel fast and slow reactions [44, 50, 51] and the fundamental observations made by Donohoe and coworkers [49] support this hypothesis.

4 Lignin Mobility and Impact on Biochemical Conversion

Lignin is a polymeric material composed of phenylpropanoid units derived primarily from three cinnamyl alcohols (monolignols): ρ-coumaryl, coniferyl, and sinapyl alcohols. Polymer formation is thought to occur via oxidative (radical-mediated) coupling between monolignols and the growing oligomer/polymer [52, 53] and is commonly believed to occur in a near-random fashion [54], although some recent studies suggest an ordered and protein-regulated lignin synthesis [55]. In any case, the resulting polymer is complex, heterogeneous, and recalcitrant to biological degradation. Although lignin loss is minimal during thermal-acidic/neutral pretreatments, it can undergo structural and chemical changes [56] that significantly influence downstream enzymatic conversion.

Although enzymes thoroughly penetrate cell walls after high severity pretreatments [49], incomplete cellulose conversion by cellulases suggests additional barriers exist at the ultrastructural level. One potential barrier is occlusion of the cellulose microfibrils by residual lignin or hemicellulose that would sterically prevent

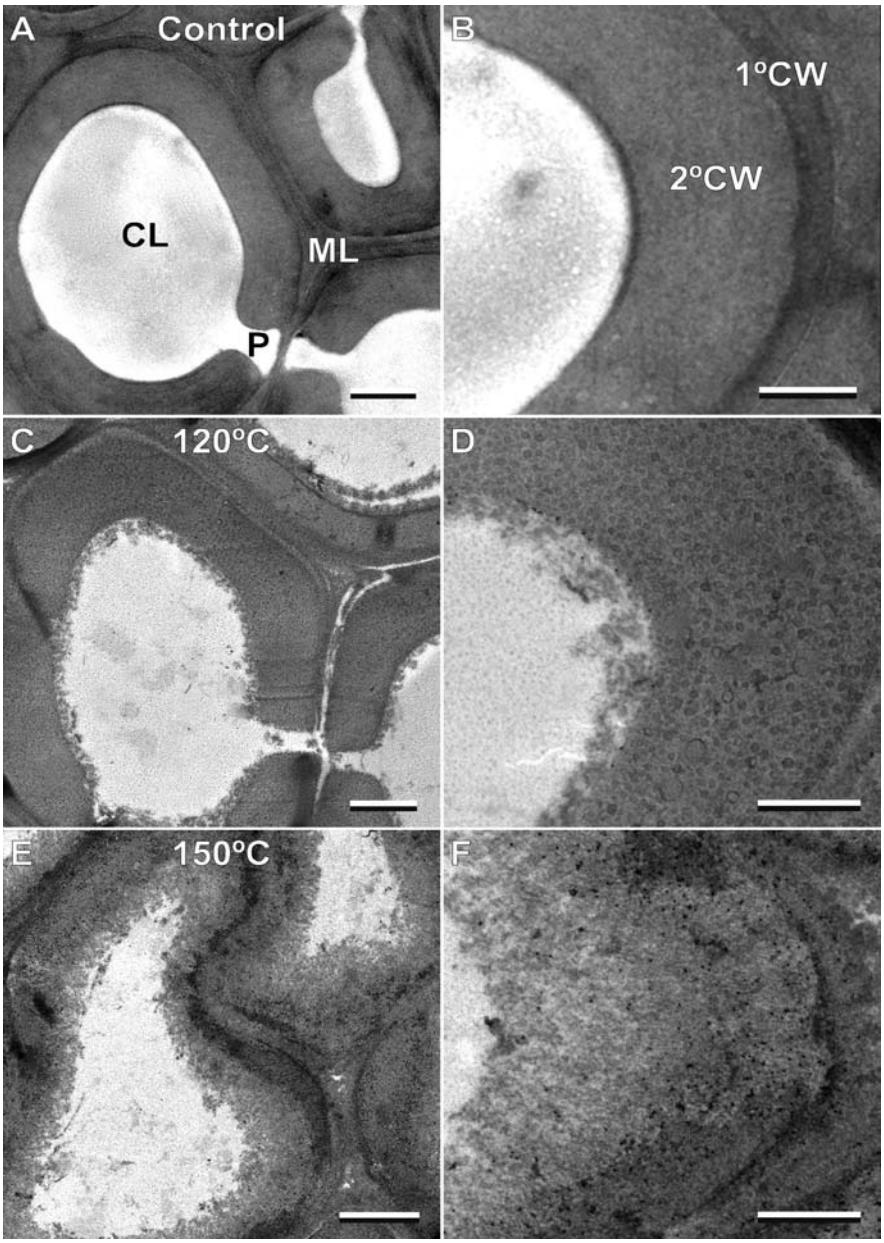

Fig. 1.3 Immuno-labeled electron micrographs of pretreated, digested corn stover cell walls. Gold particles (visible as dark dots especially in **d** and **f**) mark the location of Cel7A enzymes digesting through cell walls following dilute acid pretreatment of varying severity (120°C **c**, **d**; 150°C **e**, **f**). CL, cell lumen; ML, middle lamella; P, pit; 1° CW, primary cell wall; 2° CW, secondary cell wall. Scale bars = 1 μm **a**, **c, e**; 500 nm **b, d, f**

cellulases from binding to cellulose [42]. Other indirect mechanisms that impede complete cellulose hydrolysis are also possible such as non-productive binding of cellulases to lignin [34–36], however reports that contradict this theory also exist [57].

Enzymatic hydrolysis of biomass pretreated under alkaline conditions, which hydrolyzes less xylan than acidic pretreatments, supports the steric hindrance concept. Elevated cellulolytic activity is observed on alkaline pretreated biomass when cellulases are supplemented with xylanases and other hemicellulose degrading enzymes, likely a function of removing additional barriers to cellulose accessibility [58, 59]. A study in pretreatment variability by Selig and co-workers suggested that cellulose digestibility is improved directly by xylan removal, but only indirectly by lignin removal [47]. Removal of lignin by pretreatment appeared to increase enzymatic removal of xylan, which in turn increased cellulose digestibility. Lignin removal alone had little impact on cellulose digestion. Lignin modifying enzymes, however, have been shown to synergistically work with cellulases during digestion of steam-pretreated biomass, improving sugar yields through at least partial removal of the lignin barrier [60]. In spite of a general consensus in the scientific community about the significance of the lignin barrier to cellulose digestibility, only limited attention has been given to the fate of lignin during widely used high temperature dilute acid, hot water, and steam pretreatments which only partially remove lignin [1, 8].

A recent study investigated the fate of lignin during high temperature acid and neutral pretreatments using electron microscopy and spectroscopy techniques [40]. This study revealed that lignin could be mobilized within the cell wall matrix at temperatures as low at 120°C during both neutral and low pH pretreatments, and appears to be, at least in part, dependent on pretreatment severity. On a relatively macro scale, part of the mobilized lignin deposits back on to biomass surfaces as spherical bodies, suggesting that lignin undergoes the following sequence of events during these pretreatments – phase-transition or melting, mobilization into bulk solution, coalescence, and deposition onto solid surfaces. Scanning- and transmission electron microscopy (SEM and TEM) of pretreated cell walls shows that the lignin droplets (stained with $KMnO_4$) take a wide range of sizes (<50 nm to 2 μm) and shapes (Fig. 4a, b and Fig. 5), though the "free" shapes are uniformly spherical. Other shapes observed appear to be dictated by the physical constraints of the structures surrounding them. In addition to redeposition, there also appears to be a reorganization of lignin structure within the cell walls. A fraction of the lignin remains within the walls during pretreatment. This fraction apparently melts, but is unable to escape into the bulk liquid phase before coalescing back into droplets, as evidenced by the $KMnO_4$ stained lignin droplets that appear between layers in the cell wall (Fig. 4b–d).

Aside from the obvious implications of lignin mobility, coalescence, and redeposition observed during high temperature pretreatments, chemical modification of the lignin should also be considered. These may range from covalent bond breakage and formation to changes in inter- and intramolecular interactions. Although FTIR and NMR studies did not distinctly show chemical changes in the mobilized

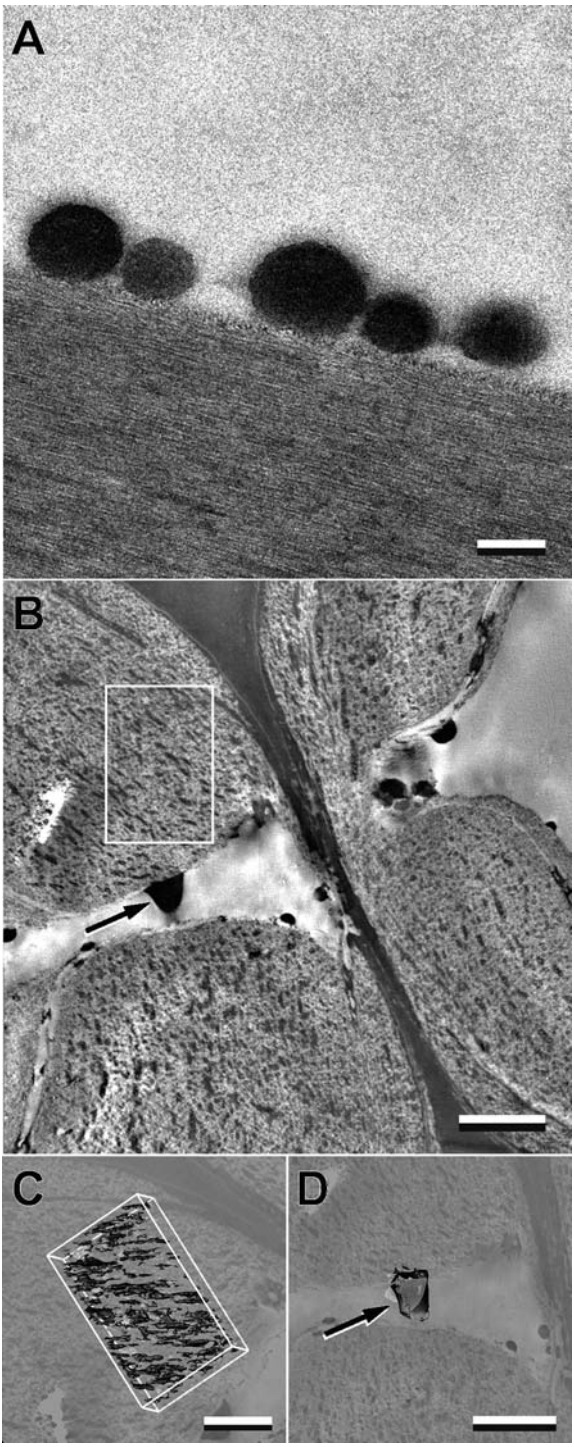

Fig. 1.4 TEM micrograph of lignin droplets re-deposited on cellulose surfaces after being transported from the cell wall matrix during high temperature pretreatments (**a**). Electron tomograph images of coalesced lignin within cell walls. The *boxed region* in **b** has been segmented to show the 3D volume of coalesced lignin (**c**). Large lignin globules can form in openings like pits (*arrow* **b**, **d**). Scale bars = 200 nm **a**; 500 nm **b**, **c**; 200 nm **d**

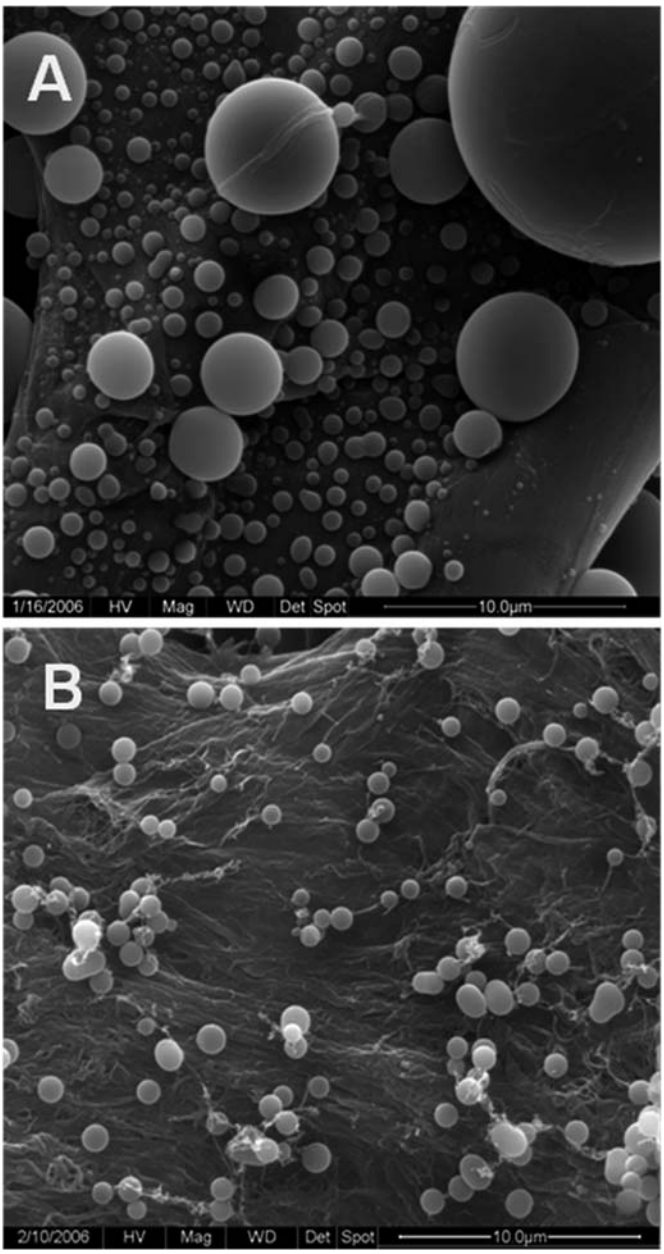

Fig. 1.5 Example SEM micrographs showing lignin droplets redeposited on to cellulose surfaces following exposure to high temperature pretreatment conditions

lignin in this study, it is possible that chemical alteration could be part of the lignin removal and transport process because lignin can partially dissolve and react in acid solutions under appropriate conditions [56]. It is further possible that part of this mobilized lignin could contain lignin-carbohydrate complexes that might sequester cellulases as observed in some studies [34, 36].

Another recent study [42] showed that purified lignin preparations as well as native lignin from corn stover could be redeposited onto clean cellulose surfaces such as filter paper. More severe pretreatments (higher temperature or acid concentrations) resulted in finer redeposited droplets. Under these conditions, digestibility of filter paper was lower by up to 15% in comparison with treatments that did not contain lignin. Since these digestions were performed at very high enzyme loadings to circumvent issues related to non-productive binding to lignin, it appears that physical blockage of the cellulose surface by lignin resulted in lower digestibility. Although redeposited lignin inhibited digestion of pure cellulose substrates in the study by Selig and coworkers [42], it is also probable that the mass transport of lignin could enhance enzymatic cellulose degradation in biomass. For example, we could visualize that as a result of lignin mass transport, the lignin sheath coating cellulose surfaces gets concentrated into droplets rendering a greater cellulose surface area available for enzymatic attack. Removal of lignin could also improve cell wall porosity allowing enzymes better access for penetration. Much work needs to be done to completely understand the nature and implications of lignin transport.

5 Rheology of Biomass Slurries and Implications for Mixing

Uniform distribution of heat, chemical catalysts, and enzymes as well as absence of product gradients within conversion reactors are all dependent on the mixing properties of biomass slurries being processed, which in turn are determined by rheological characteristics. Biomass rheology poses several challenges because of the fibrous nature of the particles, their ability to absorb water and become unsaturated at relatively low solid concentrations of 25–35% (w/w), and the continually changing particle chemical/physical properties during flow through the process. Free water content appears to be the largest factor contributing to slurry rheology. This is especially true at the high solid concentrations that are desired to make the overall process economical by lowering equipment volume and thereby cost [27]. At solid concentrations beyond the point of unsaturation, the slurries become wet granular material that agglomerate and can compact under their own weight if not adequately mixed. At lower concentrations, adequate mixing is still required to prevent settling. To further complicate matters, as biomass gets broken down into its constitutive sugars, changes occur in particle size as well as chemical properties. Water retaining polymers, such as hemicellulose and pectin, are broken down and the previously hygroscopic biomass has lower capacity for water absorption resulting in an increased amount of free water, and thereby altered slurry rheology. These dynamic changes in solid properties necessitate studies to understand rheological behavior of slurries through various process treatments.

In simplest terms, biomass slurries can be described as non-Newtonian pseudoplastic (shear-thinning) fluids [27, 61, 62]. Whereas the exact mechanism leading to pseudoplasticity in biomass slurries is unknown, a possible explanation of the behavior can be ascribed to formation of three dimensional network structure of the fibrous particles and subsequent breakdown of this structure under shear [63]. Previous studies show that while free water is present, apparent viscosity values under continuous shear increase with increasing solid concentrations. These measured apparent viscosities can be modeled with simple Casson, Bingham or Power Law models [27, 61, 62]. Thick slurries with little or no free water do not exhibit a further increase in apparent viscosity with increasing solid concentrations under continuous shear [27]. Other viscoelastic properties, such as storage and loss modulii could continue to change; however, these measurements have not yet been reported for biomass slurries.

The relatively sparse data and lack of fundamental understanding of rheological properties of biomass slurries makes calculations on mixing requirements for biomass conversion processes uncertain. Also, transport properties within biomass slurries, such as convective/conductive heat transport and convective/diffusive mass transport, and their effects on conversion are hard to discern or estimate. For example, Fig. 6 shows enzyme digestibility data obtained during digestion of pretreated corn stover at high solids pretreatments (>15% solids). Each data point was generated as a single measurement from triplicate reactors after 5 days of digestion. As can be seen from Fig. 6a, conversion of cellulose to glucose decreases steadily as solids concentrations increase suggesting inhibition of enzymes, possibly due to poor mass transfer resulting in localized accumulation of sugars as suggested by Hodge and coworkers [22]. Clearly, slurry properties will play a major role in determining these transport parameters that are crucial to determine optimal process performance across multiple scales. As another example, Fig. 7 shows experimental data from tests performed to evaluate heating time in a closed reactor containing biomass slurries of varying concentrations. These data show significant retardation of heat transfer, even with the moderate density slurries containing 10% solids (w/w). Simple heat transfer simulation models have been developed for biomass slurries assuming conductive heat transfer and a one-dimensional system; however, their validity has not been verified with experimental data [64, 65]. In unsaturated biomass slurries containing discrete aggregates, the accurate determination and prediction of transport properties might be a challenging exercise.

6 Outlook for Challenges Associated with Transport Processes in Biochemical Conversion of Lignocellulosic Biomass

Significantly greater research and development effort in the conversion of lignocellulosic biomass, spurred by economic, national security and climate change concerns over the past few years have led to significant strides in development of a fundamental understanding of transport processes that could appreciably

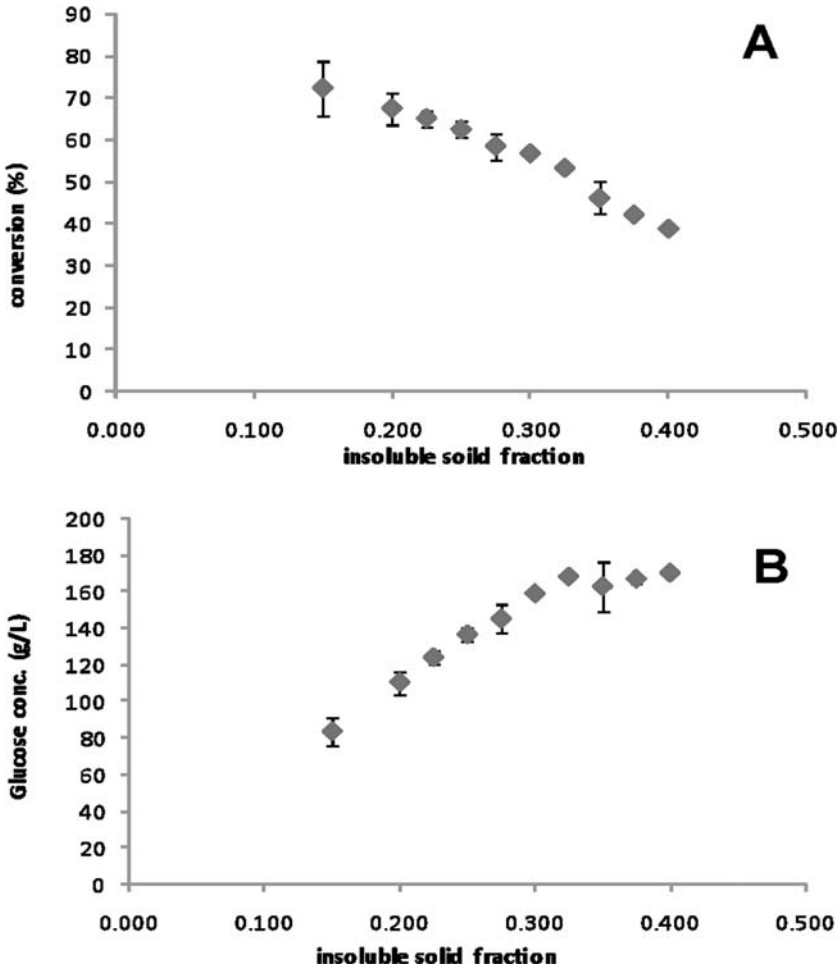

Fig. 1.6 5-day enzymatic digestibility data for pretreated corn stover showing (**a**) decrease in conversion with increasing solids concentration and (**b**) Plateau in glucose release after a solids concentration of 30%

improve overall performance and make renewable liquid transportation fuels sustainable and affordable. A thorough understanding of fundamental issues related to transport processes and the development of predictive models that integrate heat, mass and momentum transport are essential to the design, development and implementation of scale-independent processes. Continued synergism between science and engineering disciplines along with participation by industry is crucial to the development of cost-effective alternative motor fuels by 2012 and the significant displacement of fossil-derived fuels specified by the DOE (Energy Independence and Security Act of 2007) EISA for 2022. Improvements in process equipment,

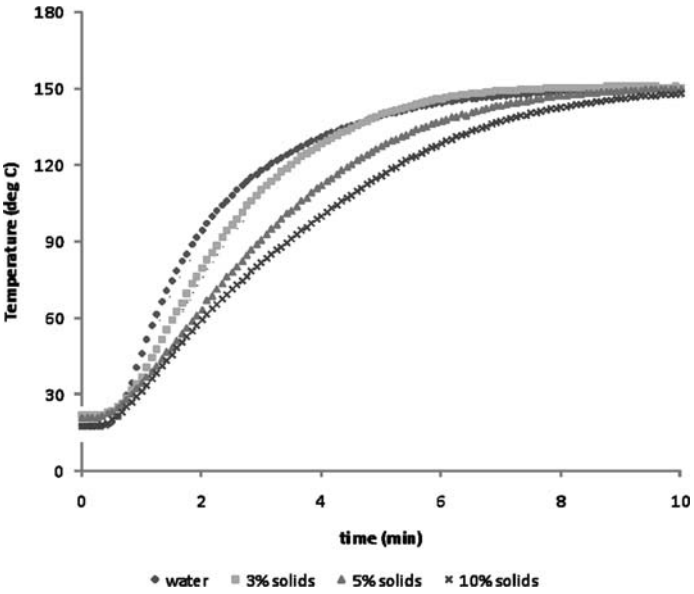

Fig. 1.7 Effect of solid concentrations on heat up time of pretreatment reactor containing biomass slurries

enzymes and microbial systems, as well as improved understanding of the basis for biomass recalcitrance are critical determinants of the successful implementation of biorefineries.

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