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Kadic, Adnan

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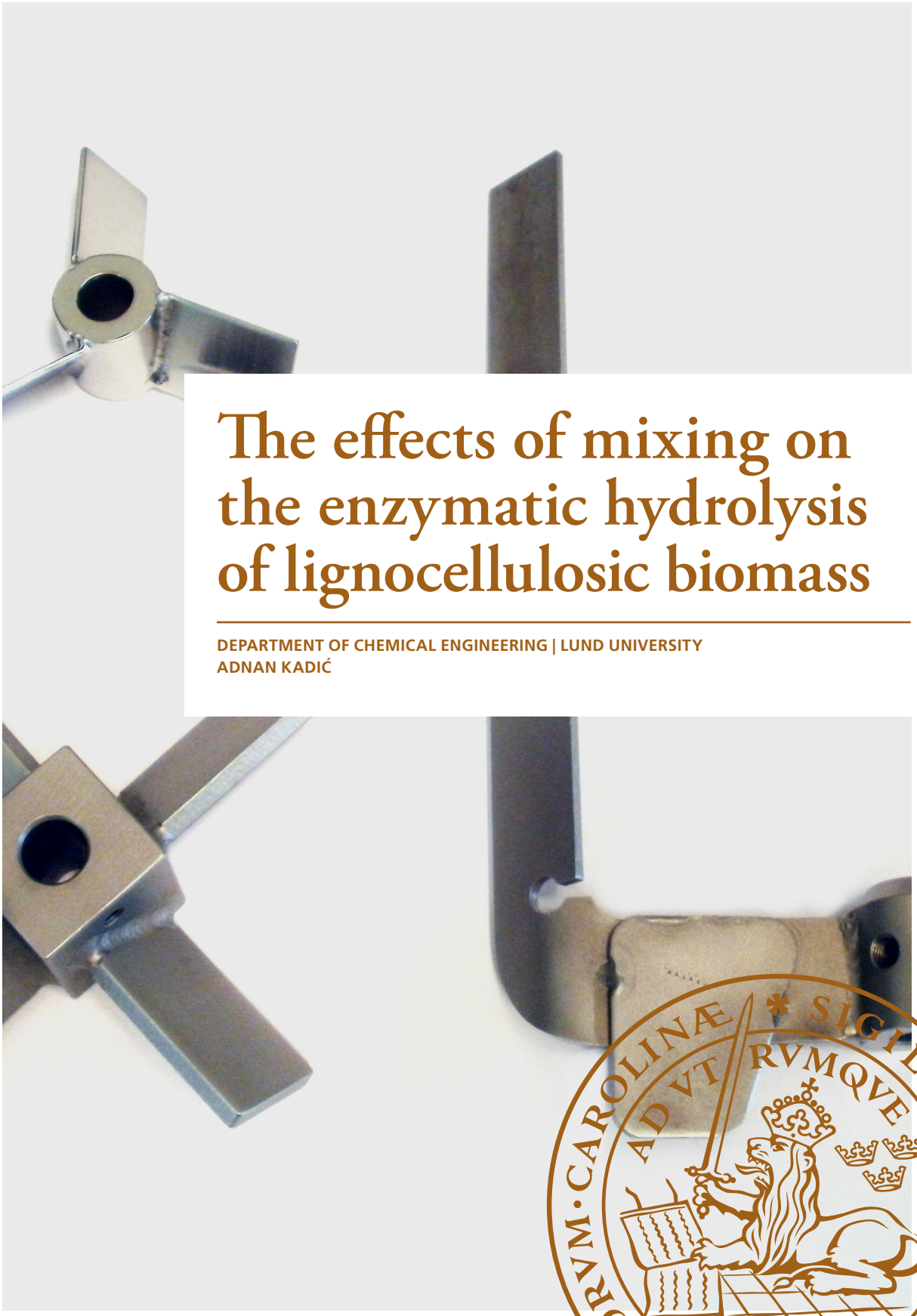

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ADNAN KADIĆ

The effects of mixing on the enzymatic hydrolysis of lignocellulosic biomass

DOCTORAL DISSERTATION
2017

Adnan Kadić

Department of Chemical Engineering
Lund University, Sweden

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The faculty opponent is Associate Professor Tina Jeoh, University of California, Davis, USA

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Abstract

Biorefining of lignocellulosic biomass into biofuels and chemicals can help replace fossil resources and decrease anthropogenic greenhouse gas emissions. This thesis is focused on the effects of mixing on the enzymatic hydrolysis of pretreated biomass. Two different types of biomass were studied: softwood (Norway spruce and Scots pine), and the energy grass giant reed. Before enzymatic hydrolysis, the biomass was pretreated by either steam or sulfite pretreatment. The first part of the work concerns the connection between particle morphology and rheology of pretreated biomass, how such properties change during the course of enzymatic hydrolysis, and how the changes are influenced by reactor mixing. The second part examines the effects of mixing in stirred tank reactors on the enzymatic hydrolysis of different pretreated materials, and also attempts to explain the mechanisms behind the observed phenomena.

The particle size reduction during enzymatic hydrolysis of steam pretreated spruce was primarily driven by reactor agitation. In the case of steam pretreated giant reed the particle size was mainly reduced by enzymatic hydrolysis. The rapid reduction in particle size of giant reed coincided with a rapid liquefaction. For steam pretreated softwood, the viscosity in fact increased at the beginning of enzymatic hydrolysis, followed by a gradual decrease during the remainder of the hydrolysis. This interesting phenomenon was in part linked to the type of pretreatment used on the softwood biomass. In contrast to steam pretreated softwood, the viscosity of sulfite pretreated spruce decreased rapidly during enzymatic hydrolysis. Efficient viscosity reduction in sulfite pretreated spruce was also achieved with very low doses of pure endoglucanase enzymes (0.1 mg protein per g glucan) without significant glucose release.

The effect of mixing on the enzymatic hydrolysis was in part determined by the viscosity of the pretreated biomass. For steam pretreated spruce at low solid loading, decreasing the agitation rate had little effect on the the enzymatic hydrolysis. However, if the viscosity was increased by the addition of a thickening agent, the effect of agitation was much larger. For a substrate that underwent rapid initial viscosity reduction, such as steam pretreated giant reed, the enzymatic hydrolysis was almost independent of agitation rate. Another important factor determining the effect of mixing on the enzymatic hydrolysis was the level of product inhibition. If the glucose and cellobiose concentrations were high, as during high solid hydrolysis of steam pretreated spruce, low agitation rate had a large negative effect on the enzymatic hydrolysis. However, if the product concentration was kept low, as during SSF, the effect of agitation was much weaker. Overall, the results indicate that the decrease in hydrolysis rate occurred due to increased local product inhibition, caused by mass transfer limitations in the stagnant zones, formed in the reactor volume when under low intensity mixing. The rate of enzymatic hydrolysis appeared to be determined by flow regime, i.e. Reynolds number, rather than specific mixing power input. This implies that the negative effects of low agitation rate will be less of a problem in larger reactors.

Key words

Softwood, spruce, enzymatic hydrolysis, mixing, viscosity

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Adnan Kadić

Department of Chemical Engineering
Lund University

LUND
UNIVERSITY

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Faculty of Engineering, Department of Chemical Engineering
Lund University, Sweden

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"All research falls under one of three categories: hard, boring or already done"

Xiao-Hui Song

Content

Abstract

Biorefining of lignocellulosic biomass into biofuels and chemicals can help replace fossil resources and decrease anthropogenic greenhouse gas emissions. This thesis is focused on the effects of mixing on the enzymatic hydrolysis of pretreated biomass. Two different types of biomass were studied: softwood (Norway spruce and Scots pine), and the energy grass giant reed. Before enzymatic hydrolysis, the biomass was pretreated by either steam or sulfite pretreatment. The first part of the work concerns the connection between particle morphology and rheology of pretreated biomass, how such properties change during the course of enzymatic hydrolysis, and how the changes are influenced by reactor mixing. The second part examines the effects of mixing in stirred tank reactors on the enzymatic hydrolysis of different pretreated materials, and also attempts to explain the mechanisms behind the observed phenomena.

The particle size reduction during enzymatic hydrolysis of steam pretreated spruce was primarily driven by reactor agitation. In the case of steam pretreated giant reed the particle size was mainly reduced by enzymatic hydrolysis. The change in particle size was different for the two materials; the area mean diameter (d_{32}) of spruce decreased from 16 to 14 μm at an agitation rate of 100 rpm, and from 16 to 12 μm at 600 rpm, both at 28% glucan conversion, while the d_{32} of giant reed decreased from 23 to 13 μm at 100 rpm and 31% conversion. The rapid reduction in particle size of giant reed coincided with a rapid liquefaction, i.e. a reduction in viscosity and yield stress of the material. For steam pretreated softwood, like spruce and pine, the viscosity in fact increased at the beginning of enzymatic hydrolysis, followed by a gradual decrease during the remainder of the hydrolysis. This interesting phenomenon was in part linked to the type of pretreatment used on the softwood biomass. In contrast to steam pretreated softwood, the viscosity of sulfite pretreated spruce decreased rapidly during enzymatic hydrolysis in a similar way as for steam pretreated giant reed. The viscosity (at a shear rate of 160.7 s^{-1}) of steam pretreated pine at 12% water insoluble solids (WIS) increased from 0.11 to 0.19 Pa·s, while for sulfite pretreated spruce at 2% WIS the viscosity decreased from 0.23 to 0.05 Pa·s, all within 15 minutes of hydrolysis. Efficient viscosity reduction in sulfite pretreated spruce was also achieved with very low doses of pure endoglucanase enzymes (0.1 mg protein per g glucan) without significant glucose release.

The effect of mixing on the enzymatic hydrolysis was in part determined by the viscosity of the pretreated biomass. For steam pretreated spruce at low solid loading (5% WIS), decreasing the agitation rate from 600 to 100 rpm, decreased the glucan conversion from 48 to 44% after 72 hours of hydrolysis. However, if the viscosity of spruce at 5% WIS was increased by the addition of a thickening agent, the effect of agitation was larger, giving a decrease in glucan conversion from 47 to 36% for the same change in agitation rate. For a substrate that underwent rapid viscosity reduction during the initial phase of enzymatic hydrolysis, such as steam pretreated giant reed, the conversion was almost independent of agitation rate. Another important factor determining the effect of mixing on the enzymatic hydrolysis was the level of product inhibition. If the glucose and cellobiose concentrations were high, as during whole slurry hydrolysis of high solid steam pretreated spruce (16% WIS), decreasing the agitation rate from 600 to 100 rpm, decreased the conversion from 38 to 17%. However, if the product concentration was kept low, as during simultaneous saccharification and fermentation, the effect of agitation was much weaker, and the glucan conversion decreased only from 49 to 42% for the same change in agitation rate. Overall, the results indicate that the decrease in hydrolysis rate occurred due to increased local product inhibition, caused by mass transfer limitations in the stagnant zones, formed in the reactor volume when under low intensity mixing. The effect of agitation remained during scale up to enzymatic hydrolysis in cubic meter size reactors. However, glucan conversion levels appeared to be determined by flow regime, i.e. Reynolds number, rather than specific mixing power input. This implies that the negative effects of low agitation rate will be less of a problem in larger reactors.

Populärvetenskaplig sammanfattning

Biomassa i form av energigrödor, och restprodukter från jordbruk och skogsbruk, är ett intressant alternativ till fossila resurser. Dessa förnyelsebara råvaror kan fås fram med lägre nettoutsläpp av växthusgaser än fossila råvaror, och i många fall till låg kostnad. Biomassan kan användas för att producera många olika produkter, till exempel bränslen och viktiga kemiska byggstenar, i anläggningar liknande dagens oljeraffinaderier, s.k. bioraffinaderier. De huvudsakliga beståndsdelarna i biomassa är cellulosa och hemicellulosa, dvs. sockerpolymerer, vilket gör enkla socker, som t.ex. glukos, till en naturlig utgångspunkt i ett bioraffinaderi. I ett bioraffinaderi behöver biomassan först ”luckras upp” i ett förbehandlingssteg, innan cellulosan kan enzymatiskt brytas ned till glukos. När det gäller den enzymatiska hydrolysen så är det önskvärt att uppnå en hög nedbrytningsgrad av cellulosan utan att tillsätta för mycket enzymer. Efter hydrolysen kan de enkla sockerarterna omvandlas mikrobiellt till olika kemikalier genom jäsning. Det möjliga produktutbudet från hela processen är mycket brett, med olika typer av transportbränslen, organiska kemikalier, el och värme. För att minska processkostnaderna är det önskvärt att minska mängden vatten som går igenom processen, vilket innebär att biomassan bör hanteras vid hög torrhalt. Problem uppstår då på grund av reologin, dvs. flödesegenskaperna, av förbehandlad biomassa vid hög torrhalt. Den höga viskositeten gör det svårt att blanda om och pumpa materialet utan mycket höga energikostnader. Dessutom kan dålig omblandning ha en negativ inverkan på den enzymatiska hydrolysen av cellulosa och leda till ett lågt utbyte.

Målet för denna avhandling är att förstå effekterna av omblandning på enzymatisk hydrolys av cellulosa. För att förstå omblandning var det nödvändigt att studera hur typen av biomassa och förbehandling bestämmer reologin, och hur reologin påverkas av enzymatisk hydrolys. Det visade sig att låg omrörning minskade graden av enzymatisk hydrolys av ångförbehandlad gran vid hög torrhalt. Detta var troligen kopplat till den höga viskositeten av materialet, och det faktum att viskositeten minskade relativt långsamt under hydrolysens gång. Effekten av omrörningen berodde på flödesegenskaperna i reaktorn, och inte energiåtgången för omrörningen, vilket är en positiv slutsats, eftersom det är lättare att uppnå ett lämpligt flöde i en storskalig hydrolysreaktor. Ett annat sätt att minska de negativa effekterna av dålig omrörning visade sig vara att jäsa glukosen samtidigt som den

frisläpps under hydrolysen. Omrörningen hade ingen effekt på hydrolysen av ångförbehandlad *Arundo donax*, en slags gräsliknande energigröda, sannolikt pga. att materialets viskositet minskade mycket snabbt under hydrolysens gång. Reologin av ett material beror inte bara på typen av biomassa, utan också på typen av förbehandling. Genom att förbehandla gran på ett annat sätt, som liknar sulfitprocessen i pappersindustrin, blev det mycket lättare att reducera viskositeten av materialet med hjälp av enzymer, vilket också underlättade omblandning.

List of publications

This thesis is based on the following publications, which will be referred in the text by their Roman numeral:

I. **Kadić A**, Palmqvist B, Lidén G. (2014) Effects of agitation on particle-size distribution and enzymatic hydrolysis of pretreated spruce and giant reed. Biotechnol Biofuels 7:77

II. **Kadić A**, Lidén G. Viscosity reduction of pretreated softwood by endoglucanases. (*Manuscript*)

III. Palmqvist B, **Kadić A**, Hägglund K, Petersson A, Lidén G. (2016) Scale-up of high-solid enzymatic hydrolysis of steam-pretreated softwood: the effects of reactor flow conditions. Biomass Conv Bioref 6:173–180

IV. **Kadić A**, Lidén G. (2017) Does sugar inhibition explain mixing effects in enzymatic hydrolysis of lignocellulose? J Chem Technol Biotechnol 92:868–873

My contributions to the publications

I. I participated in the design of the study, performed the experimental work and wrote the manuscript.

II. I designed the study, performed the experimental work and wrote the manuscript.

III. I participated in the design of the study and the laboratory experimental work. I was involved in the preparation of the manuscript.

IV. I designed the study, performed the experimental work and wrote the manuscript.

Abbreviations

1G – 1st generation

2G – 2nd generation

CBH – Cellobiohydrolase

CBM – Carbohydrate-binding module

DM – Dry matter

DP – Degree of polymerization

EG – Endoglucanase

GHG – Greenhouse gas

HPLC – High-performance liquid chromatography

LPMO – Lytic polysaccharide monooxygenase

NREL – National Renewable Energy Laboratory

PSD – Particle size distribution

SSF – Simultaneous saccharification and fermentation

STEX – Steam explosion

WIS – Water insoluble solid

1. Introduction

1.1 Background

Global warming caused by anthropogenic emissions of greenhouse gases (GHG), mainly carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O), has been recognized as one of the main challenges facing humanity in the 21st century [1]. The annual global CO_2 emissions (from fossil fuel burning and cement production) were estimated to be 35 Gt in 2011. As of 2011 the cumulative CO_2 emissions (since 1751) reached 1,400 Gt, of which 70% were released after 1970 [2]. In the period of 1870–2013 the US and EU(28) contributed to 49% of the emitted CO_2 [3]. If current environmental policies are implemented, the global CO_2 emissions are projected to reach 43 Gt in 2040, raising the cumulative emissions to approximately 2,500 Gt at that time [4]. Most of the anthropogenic GHG emissions can be attributed to electricity generation, industry and land use (70% of 2010 emissions), while transportation is the largest remaining sector contributing to 14% [5].

The single most important consequence of anthropogenic GHG emissions is the increase in the temperature of Earth's climate system. According to the GISS (Goddard Institute for Space Studies) temperature analysis scheme, the global mean surface temperature has increased by 0.86 ºC as of 2015, relative to the 1951–1980 mean [6,7]. If the global temperature is to be stabilized (66% probability of keeping warming below 2 °C) less than 1,200 Gt of anthropogenic CO_2 can be emitted from 2015 and onwards [3]. Current energy policies around the world *call in question* the likelihood of such a scenario, and the global temperature may increase by more than 2 °C [1]. The rise in atmospheric temperature and CO_2 levels is predicted to lead to a myriad of consequences, including e.g. rising sea levels [8], ocean acidification [9], changing precipitation patterns [10], and extreme local temperatures [11], with adverse effects on Earths biota and humanity itself. In order to reduce the probability of negative climate change outcomes, a wide variety of measures have been proposed, such as improved energy efficiency, application of low-GHG energy sources for electricity generation (solar, wind, hydro, bio and nuclear energy), the use of "carbon neutral" energy/raw-materials in industry, positive land use changes (afforestation) and electrification or biofuel use in the transport sector [5].

1.2 Biofuels and Biochemicals

The biorefinery - an industrial facility for production of bioenergy, biofuels and biochemicals from biomass [12] - stands out as a solution that can reduce GHG emissions from several different sectors of the economy, and if combined with good land management [13] and carbon capture and storage [14], it can be used to sequester atmospheric carbon.

Many different biofuels, such as bioethanol, biodiesel, biogas and biobutanol can serve as replacements for fossil derived transportation fuels. Currently, bioethanol is the biofuel produced in the largest volumes, with a global production 97.2 billion liters in 2015. The US and Brazil are the largest producers, with annual outputs of 56 and 27 billion liters, respectively [15]. Most of the ethanol is produced by fermentation of easily accessible '1st generation' (1G) sugars, i.e. glucose derived from corn starch, or sucrose from sugarcane juice. However, concerns about the sustainability of 1G ethanol production have been raised. The GHG emissions from bioethanol vary due to many factors, including type of feedstock and source of process energy. Overall, 1G ethanol is considered to provide moderate reduction in GHG emissions [16,17]. Competition between 1G ethanol and food production, and its effect on food security and GHG emissions, is another issue of concern [18].

In contrast to starch and sucrose, cellulose is not a food source for humans, and together with lignin and hemicellulose, it makes up most of the stems and leafs of wood and agricultural crops. So called "2nd generation" (2G) ethanol, i.e. ethanol from lignocellulosic sugars, has been put forward as one possible solution to the sustainability issues surrounding current bioethanol production. As a result, development of 2G ethanol has been given political and financial support in the EU and the US. The Renewable Energy Directive adopted by the EU in 2009 set a target for 10% of transport fuels from renewable sources by 2020 and put forward a biofuel sustainability criterion [19]. Financial support for the development of 2G ethanol has been provided through several programs, including the 7th Framework, Horizon 2020 and NER300 [20]. One such EU supported action was the development of the first commercial scale 2G ethanol plant in Crescentino, Italy, which opened in 2013. The plant, owned by Beta Renewables, has a nominal annual capacity of 50 million liters of ethanol.

In the US, the Energy Independence and Security Act of 2007 set direct volume targets up to 2022 for different renewable fuels, including cellulosic biofuel [21]. Financial support for the development of 2G ethanol has also been provided by the Department of Energy. In 2014–2015, three large cellulosic ethanol plants came

online in the US operated by POET-DSM, Abengoa Bioenergy[1] and DuPont, with a combined annual capacity of 290 million liters. However, the total production of cellulosic biofuels in the US was a mere 12 million liters in 2016, falling way short of the 58.7 billion liter target set for 2022 [22]. The slow commercialization of cellulosic ethanol indicates that significant technological challenges still remain. Additionally, there are concerns about the suitability of ethanol as a replacement transportation fuel. For example, only lower blends of ethanol (< 15%) have been approved for use in non-modified gasoline engines in the US [23]. Furthermore, ethanol cannot be used as a drop-in fuel in diesel engines. Thus, effort has been devoted towards R&D on 2G drop-in fuels, including the Horizon 2020 project ButaNext on butanol [20] and DOE funded projects on "biohydrocarbons", such as farnesene [24].

Following heat/electricity and transportation fuels, the plastics industry is the largest consumer of oil and natural gas, with the global plastics production reaching 320 million metric tons in 2015 [25]. There is significant potential for plastics derived from sugars, since it is possible to produce several useful monomers by fermentation. Examples include lactic acid, succinic acid, 1,4-butanediol, 1,3-propanediol and ethylene, which is obtained from ethanol through dehydration [26]. Recently, there has been an upswing in commercial production of plastics and plastics precursors from 1G sugars, including PLA from corn starch (140,000 t/year) by NatureWorks, 1,3-propanediol from corn starch (120,000 t/year) by DuPont Tate & Lyle, polyethylene from sugarcane (200,000 t/year) by Braskem and succinic acid from corn starch (30,000 t/year) by Bioamber [27]. However, as with 1G bioethanol, there are concerns about the sustainability of plastics produced from 1G sugars. Shifting the feedstock to lignocellulose based sugars can thus also be of interest in this sector.

1.3 Scope and outline of the thesis

The biochemical conversion of lignocellulosic biomass into liquid biofuels or biochemicals is completed in four main process steps: biomass pretreatment, enzymatic hydrolysis, fermentation and product recovery. The research work presented in this thesis concerns the initial process steps, i.e. pretreatment and enzymatic hydrolysis. The work can be divided into two main parts. The first part deals with the particle size and rheology of pretreated biomass. This topic has implications for multiple biorefinery operations, such as pretreatment, viscosity reduction and enzymatic hydrolysis. The second part focuses on the enzymatic

[1] Ceased production December 2015 due to financial difficulties [181]

hydrolysis *per se* and explores the effect of mixing on the conversion of cellulose in pretreated biomass to glucose.

The aim of the work was to:

- characterize the effect of reactor agitation on the particle size and rheology of pretreated biomass
- understand the effect of mixing on the enzymatic hydrolysis of lignocellulose

These are central issues, which affect both process design and process economy in biorefineries.

The structure of the thesis is as follows. Chapter 2 gives a background overview of the structure and composition of biomass, and how it is processed through a generic 2nd generation biochemical biorefinery. The remaining sections directly concern the work done in the current study. In Chapter 3 changes in particle size distribution and viscosity as a result of agitation and enzymatic hydrolysis, for different types of biomass, such as steam pretreated spruce, pine and giant reed, and sulfite pretreated (delignified) spruce are discussed, whereas in Chapter 4 the effects of agitation and scale-up on enzymatic hydrolysis of lignocellulose are presented. Factors, such as stagnant zones and mass transfer limitations are analyzed in relation to conversion of cellulose. Finally, Chapter 5 summarizes the main results of this work and suggests paths for future research.

2. Biomass and the Biorefinery

"Biorefinery" is a relatively broad, and sometime ambiguous, term. In the present text the term "biorefinery" is used to denote a facility that processes biomass into various products, such as biofuels and biochemicals, and may also produce excess heat and electricity to be sold on the energy market. "Biomass" in its most general sense includes organic matter from all living organisms, but in the context of the biorefinery, biomass normally refers to plant materials and algae. The range of plant materials that can be used is very wide and includes for example sugar crops, oil crops, cereals, straw, energy crops, softwoods, hardwoods and macro- and micro- algae.

According to the biorefinery classification developed within International Energy Agency Bioenergy Task 42, biorefineries can be classified according to platforms, products, feedstocks and processes [28]. The sugar platform involves the production of C_5 and/or C_6 sugars as an intermediate step, and is usually a biochemical process [28]. If lignocellulosic biomass, such as crop residues, softwoods and hardwoods, is used as the feedstock, it is considered as a 2nd generation (2G) biorefinery.

This dissertation focuses on the enzymatic hydrolysis of lignocellulose to sugars within the framework of a biochemical 2G biorefinery. Chapter 2 is intended to provide an overview of the structure of lignocellulosic biomass, how cellulose can be broken down by enzymatic hydrolysis, and how this process can be utilized within the context of the biorefinery.

2.1 Biomass structure

Lignocellulose, consisting primarily of cellulose, hemicellulose and lignin, makes up most of the stems and leafs of terrestrial plants. It is primarily found in the xylem, i.e. the inner part of the stem. While woody plants have very thick stems, most grasses are annuals with relatively thin stems. Plant cells in the xylem have multi-layered cell walls, comprised of a thin outer layer referred to as the middle lamella, the primary cell wall, and the secondary cell wall. The middle lamella (ML) is highly lignified and ensures adhesion between the plant cells. Primary cell

walls are thin and consist mostly of cellulose, pectin and hemicellulose [29]. The thick secondary cell wall, made up of lignocellulose, is thought to consist of three layers, S_1, S_2 and S_3, with differing cellulose orientation. The outer S_1 and S_3 layers are relatively thin, with the thick inner S_2 layer providing most of the mechanical strength of the fiber [30]. The lumen (L) of living plant cells is surrounded by a cell membrane. A schematic image of the microstructure of the wood cell wall is shown in Figure 2.1. The microstructure is not uniform along the entire length of the fiber. For example, regions of increased structural disorganization, i.e. dislocations, occur at repeated intervals on the fiber [31].

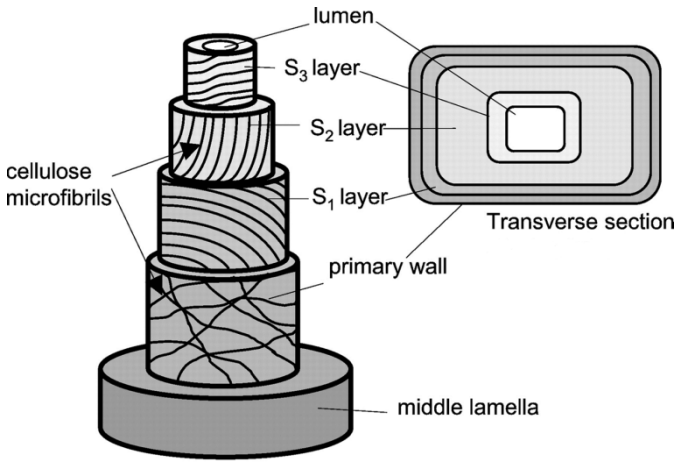

Figure 2.1. Schematic representation of the microstructure of the wood cell wall. Image adapted from [30].

Cellulose in terrestrial plants is synthesized by large cellulose synthase (CES) complexes in the plasma membrane, consisting of 12–36 CES proteins, arranged in a hexagonal "rosette". Each CES protein elongates one cellulose chain by a simultaneous addition of two UDP-glucose units. Each UDP-glucose is rotated by 180º, making cellobiose (linked by β-1,4-glycosidic bonds) the repeating unit of the cellulose polymer [32].

There is significant variation in the reported cellulose chain length, i.e. degree of polymerization (DP), even for the same plants. This is likely due to difficulties in isolating intact cellulose from complex plant tissues. The DP values reported in the literature are generally in the range of 1,000–10,000, with the cellulose chains in woody plants being somewhat longer than in grasses [33,34]. Cellulose chains leaving the CES complex assemble into microfibrils. Within the microfibril the

chains are held together primarily by hydrogen bonds and hydrophobic interactions, thus forming the rod-like structure of the microfibril [35–38].

The structure of microfibrils in different plant species is difficult to determine due to the complexity of plant tissues. The cross-section of microfibrils is thought to be rectangular or diamond shaped, with the thickness varying significantly between different plant species; e.g. microfibrils of up to 1,000 cellulose chains have been observed in micro-algae [39]. Spruce wood microfibrils most likely have a rectangular cross-section, comprised of 24 parallel cellulose chains, with a thickness of about 3 nm [40]. In wood, there is evidence of aggregation of microfibrils into larger structures referred to as fibrils with a thickness of 10–20 nm [41].

Cellulose is known to have crystalline properties; however, due to the complexity of the lignocellulose matrix, the interpretation of the crystallinity measurements is not simple. Different crystalline allomorphs of cellulose have been identified, with cellulose I (α and β) being the predominant forms found in nature [39]. However, native cellulose samples are rarely entirely crystalline, which has been attributed to the existence of an additional form of amorphous cellulose. Overall, the observations suggest that the interior of the microfibrils is truly crystalline, whereas the external cellulose chains, in addition to cellulose chain ends, represent most of the "amorphous cellulose" observed in crystallinity measurements [39].

In addition to cellulose, several other polysaccharides are present in lignocellulose. These are not always easy to classify due to their chemical and structural complexity. Most can be assigned to the category of hemicelluloses, defined as polysaccharides with a backbone consisting of β-1,4-linked glucose, mannose, and/or xylose, with also other sugars (and sugar acids) sometimes being part of side chains [42]. Hemicellulose plays a role in improving the structural stability of lignocellulose by linking together microfibrils through hydrogen bonding. The most abundant forms of hemicellulose in the secondary cell wall of grasses, softwoods and hardwoods are glucuronoarabinoxylan, galactoglucomannan and glucuronoxylan, respectively [42]. The shorter chain length of hemicellulose (DP ~ 80–200) allows for intracellular synthesis of the polysaccharide in the Golgi apparatus, after which the chains are transported to the plasma membrane [43].

The third major biopolymer in lignocellulose is lignin, which serves to improve the structural stability of lignified plant tissues, and enables the transport of water through the plant vascular system by increasing the hydrophobicity of the cellulose matrix. Lignin is formed by the polymerization of the monolignol alcohols p-coumaryl, coniferyl and sinapyl, which correspond to the p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) phenylpropanoid units, respectively, when incorporated into lignin. The monolignol alcohols are synthesized in the cytoplasm by the phenylpropanoid pathway, and transported across the plasma membrane by

ATP-binding cassette-like transporters [44]. The alcohol monomers are then cross-linked via radical–radical coupling by oxidative enzymes to form lignin macromolecules [45]. The composition of lignin varies between different plant species; hardwood lignins consist mostly of G and S units, softwood lignins are composed almost entirely of G units, and lignins from grasses incorporate G and S units at comparable levels, with appreciable amounts of H units. A schematic image of the nanostructure of the wood cell wall, including cellulose fibrils, hemicellulose and lignin, is shown in Figure 2.2.

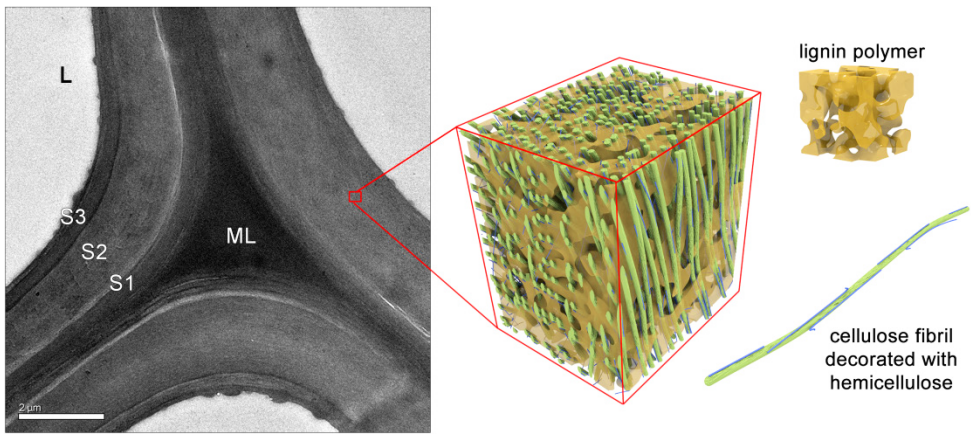

Figure 2.2. (Left) Transmission electron microscope image showing the microstructure of the wood cell wall. (Right) Schematic representation of the nanostructure of the wood cell wall. Images courtesy of Peter Ciesielski, adapted from [46].

The composition of the biomass determines the distribution of the simple sugars formed in the degradation of biomass. The differences in composition between softwoods, hardwoods and grasses (crop residues and energy crops) are significant (Table 2.1). The high glucan and lignin content in wood, due to the predominance of secondary cell walls, and the abundance of mannan in softwoods and xylan in grasses, are notable.

Table 2.1. Composition of lignocellulosic biomass (% dry mass; n.r. - not reported)

	Glucan	Mannan	Galactan	Xylan	Arabinan	Lignin
Softwoods						
Spruce [47]	43.8	14.5	n.r.	6.3	n.r.	28.8
Pine [48]	43.6	10.8	2.2	6.6	1.6	26.8
Hardwoods						
Willow [49]	43.0	3.2	2.0	14.9	1.2	26.6
Poplar [50]	43.8	n.r.	n.r.	14.8	n.r.	29.1
Oak [47]	45.2	4.2	n.r.	20.3	n.r.	24.3
Crop residues						
Wheat straw [51]	30.2	n.r.	0.8	18.7	2.8	17.0
Corn stover [52]	38.3	n.r.	2.1	21.0	2.7	17.4
Rice straw [53]	31.1	n.r.	n.r.	18.7	3.6	13.3
Sugarcane bagasse [54]	43.0	n.r.	0.4	26.0	1.5	24.6
Energy crops						
Switchgrass [55]	39.5	n.r.	2.6	20.3	2.1	21.8
Giant reed [56]	35.7	0.2	0.6	18.6	1.6	22.3

In this thesis, three different types of biomass were used; the two softwoods Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris* L.), and the perennial grass giant reed (*Arundo donax* L.). The two softwood species were of interest as representative of the most common species in the Scandinavian forestry, whereas *Arundo donax* is an example of a high yielding crop, which is said to be able to grow on secondary lands. For that reason, it has been of interest as a feedstock for the 2G ethanol plant in Italy operated by Beta Renewables [57].

The structure of the feedstocks used was very different. No wood is formed in grasses [30], i.e. the xylem is thin and the fibers are not tightly associated. This can affect fiber separation and shortening during pretreatment and hydrolysis. The particle/fiber morphology in turn determines the rheology of the pretreated biomass, and thus the quality of the mixing during enzymatic hydrolysis. As discussed later in this thesis, the properties of the biomass have a profound effect on the design of pretreatment, liquefaction and mixing in the lignocellulosic biorefinery.

2.2 The Biorefinery

The biochemical 2G biorefinery comprises four main process steps: biomass pretreatment, enzymatic hydrolysis, fermentation and product recovery. In a typical "energy focused" biorefinery (Figure 2.3) the main purpose of the pretreatment is to increase the enzymatic digestibility of the biomass. Following pretreatment, all components of the biomass, including lignin, hemicellulose sugars and cellulose, are taken through the enzymatic hydrolysis, fermentation and product recovery steps. One main product, usually ethanol, is recovered. The remaining stream from the product recovery is a complex mixture, i.e. a solid fraction consisting of lignin, residual cellulose, protein and yeast, and a liquid fraction containing remaining sugars and sugar degradation products, organic acids, phenolic compounds and soluble lignin. Finally, the energy value of this stream is recovered by combustion and/or anaerobic digestion of the solid and liquid fraction.

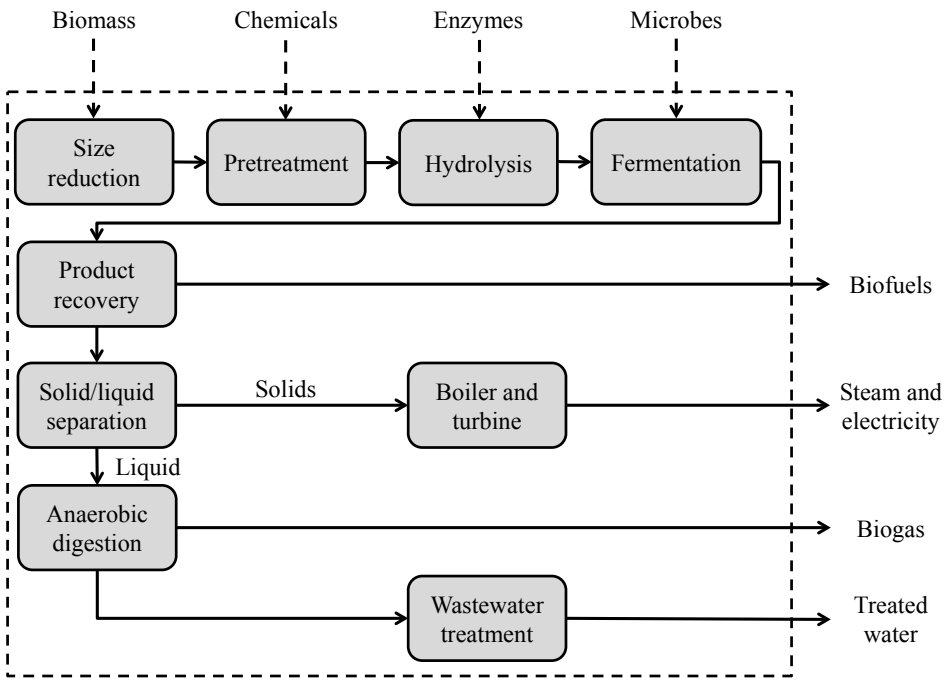

Figure 2.3. Overview of the process steps in an "energy focused" biorefinery.

In a biorefinery aimed at recovery of pure lignin and sugars, i.e. a "valorization focused" biorefinery (Figure 2.4), the process layout is somewhat different. Here, the purpose of the pretreatment is not only to increase the enzymatic digestibility of the cellulose fraction, but also to recover a relatively pure lignin fraction. Overall, this entails a different process layout less focused on energy generation.

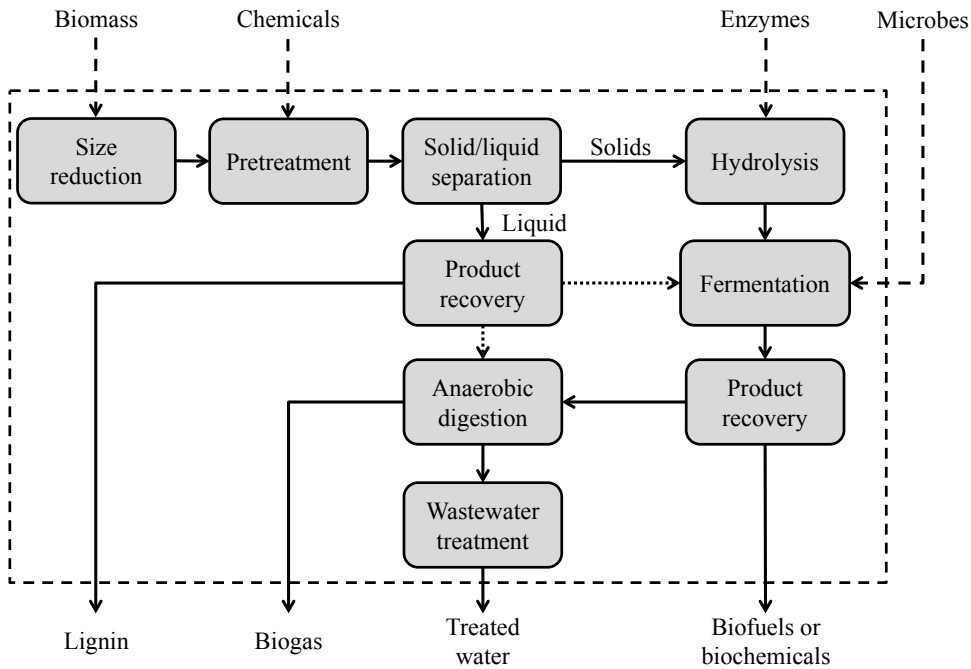

Figure 2.4. Overview of the process steps in a "valorization focused" biorefinery.

The remainder of Chapter 2 will provide an overview of the initial steps, i.e. the steps directly related to the release of lignocellulosic sugars from the biomass - the pretreatment and enzymatic hydrolysis. The section on pretreatment will center on the forms of pretreatment used in this thesis, framed in the context of the "energy focused" or "valorization focused" biorefinery. The section on enzymatic hydrolysis will provide an overview of the biochemistry of cellulose hydrolysis.

2.2.1 Pretreatment

Any perennial plant must be able to resist a fast microbial degradation from cellulolytic organisms. Outer layer protection in terms of the bark is important, but

also the very structure of lignocellulose makes it recalcitrant to the activity of cellulolytic enzymes. In order to improve cellulose hydrolysis in a technical process it is common to apply a so-called “pretreatment” step, where “pre-” can be understood from the fact that it precedes the enzymatic hydrolysis. Pretreatment modifies one or more properties of the biomass that are thought to affect cellulose hydrolysis, such as crystallinity, degree of polymerization, cellulose accessibility, lignin and hemicellulose content [58].

The simplest form of pretreatment is mechanical comminution, which involves chipping, grinding, and/or milling, i.e. particle size reduction, of the biomass. Particle size reduction to the micron range (1–1,000 μm) can improve the enzymatic hydrolysis of different kinds of biomass, such as softwood [59,60], and to a lesser extent crop residues, such as corn stover [61], wheat straw [62] and sugarcane bagasse [63]. The main objection raised against the practical application of mechanical comminution as a form of pretreatment is the high energy expenditure. The energy requirement for coarse size reduction (0.5–5 mm) of wood biomass is 80–100 W·h per kg of dry wood [64]. However, particle size reduction in the micron range is much more energy intensive and requires 500 W·h per kg of dry wood [59].

The most common forms of pretreatment utilize elevated temperature (160–230 ºC) in order to increase the rate of hydrolysis in water solution. Steam pretreatment and hydrothermal pretreatment belong to this category. If the material is injected with high pressure saturated steam, the method is usually referred to as steam pretreatment [65]. During batch steam pretreatment it is possible to carry out rapid pressure reduction, i.e. explosive decompression. This is commonly referred to as steam explosion (STEX). Although the explosion aspect plays a role in fiber fragmentation and separation, it seems to be less important for the enzymatic digestibility of the material [66]. In the case of hydrothermal pretreatment, the biomass is soaked or washed with pressurized hot water [67,68].

Both steam pretreatment and hydrothermal pretreatment hydrolyze, at least partially, the hemicellulose fraction of the biomass, even without added acid catalyst [67,69]. This occurs by autocatalysis, i.e. hydronium ions from water at elevated temperature and acidic compounds released from the biomass, such as acetic acid, catalyze the hydrolysis [70]. At a given temperature and residence time, the hemicellulose hydrolysis may be further improved by the addition of an acid catalyst, such as sulfur dioxide or sulfuric acid [69,71]. The addition of acid can also improve the enzymatic digestibility of the remaining cellulose fraction [69,72], particularly in the case of sulfur dioxide catalyzed steam pretreatment of softwood [73–75]. In the case of dilute acid hydrolysis (DAH), the pretreatment is usually performed at lower temperature (< 160 ºC) and longer residence time (20–60 min) [76,77]. While removing most of the hemicellulose, steam and

hydrothermal pretreatment do not cause significant delignification of the biomass [69,78]. These forms of pretreatment are thus more suitable for the "energy focused" biorefinery (cf. Figure 2.3). Due to cost effectiveness and relative simplicity, steam and hydrothermal pretreatment have been incorporated in several pilot and demonstration facilities around the world.

In the case of the "valorization focused" biorefinery, the purpose of the pretreatment is not only to improve the enzymatic digestibility of the cellulose, but also to recover a relatively pure lignin fraction (cf. Figure 2.4). In this case other forms of pretreatment are suitable, such as ammonia recycle percolation [79,80], organosolv [81,82] or sulfite pretreatment [83,84]. These types of pretreatment provide an additional product, in the form of extracted lignin or water soluble lignosulfonate, and significantly improve the enzymatic digestibility of the solid cellulose fraction. They may be especially suitable for recalcitrant biomass, such as softwood, on which the performance of steam pretreatment has been less impressive [85]. A negative aspect of these types of pretreatment is the increased complexity, as they may require washing and dewatering of the cellulose fraction in order to recover lignin, and in some cases additional process steps for solvent recovery.

In this thesis several forms of biomass pretreatment were used. The perennial grass giant reed was steam pretreated (no catalyst added) at the Rivalta Scrivia R&D Center (Biochemtex S.p.A. Italia, Rivalta, Italy) [86]. Two types of softwood, Norway spruce and Scots pine, were pretreated by SO_2 catalyzed steam pretreatment. In this case the pretreatment conditions were as follows: temperature of 210 ºC, residence time of 5 min and SO_2 loading of 2.5% based on biomass moisture content. The Scots pine was pretreated in a 10 L batch steam pretreatment reactor [87] at the Process Development Unit (Lund University, Sweden). The Norway spruce was pretreated in a continuous steam pretreatment unit at the Biorefinery Demo Plant (Örnsköldsvik, Sweden) [88]. Norway spruce was also pretreated using sulfite pretreatment at Borregaard AS (Sarpsborg, Norway). The technology used was similar to a previously described method developed by Borregaard AS [89], [90].

2.2.2 Enzymatic hydrolysis

The purpose of the pretreatment in a biochemical process is not to directly solubilize or hydrolyze cellulose to any appreciable extent, but rather to facilitate the subsequent enzymatic hydrolysis. Enzymatic hydrolysis of cellulose was first observed in the 1900s, when G. Seillière demonstrated the hydrolysis of regenerated cellulose by crude snail enzyme isolate [91]. The mode of action of cellulases was for a long time unknown. However, in the 1950s, T. E. Reese proposed the C_1-C_x model, which postulated the existence of C_1 enzymes that make native crystalline cellulose susceptive to the hydrolytic C_x activity [92]. The isolation of the individual enzymes in the 1970s introduced the idea of two major types of cellulases; endoglucanases (EG) that cut the cellulose chain, and processive exoglucanases, later referred to as cellobiohydrolases (CBH), that degrade the cellulose from the chain ends [93]. The advent of sequence and structural data in the 1990s has led to the further more detailed classification of cellulases into different glycoside hydrolase (GH) families shedding light on the substrate interactions and catalytic mechanisms of the common cellulases [94]. Glycoside hydrolases, according to the enzyme commission nomenclature, belong to category EC 3.2.1.-, i.e. hydrolases acting on sugars and hydrolyzing O- and S-glycosyl compounds.

Commercial cellulases are based on enzymes secreted by *Trichoderma reesei* (named in honor of T. E. Reese), which is the asexual form of the fungus *Hypocrea jecorina*. This strain secretes multiple GHs, including two cellobiohydrolases and six endoglucanases [94]. The secretome of *T. reesei* is dominated by the GH7 cellulase CBH I (Cel7A) [95]. CBH I carries out processive hydrolysis from the reducing end of cellulose, with cellobiose being the main hydrolysis product [96,97]. The other cellobiohydrolase secreted by *T. reesei*, CBH II (Cel6A), belongs to the GH6 family and hydrolyzes cellulose from the nonreducing end [98]. The main endoglucanases secreted by *T. reesei* are EG I (Cel7B), belonging to the GH7 family, and EG II (Cel5A) from the GH5 family [95]; these enzymes contribute 25% and 55%, respectively, of the total EG activity in *T. reesei* [99]. Overall, CBH I/II and EG I/II represent more than 90% of the cellulases secreted by *T. reesei* [100]. Another important class of enzymes needed for complete hydrolysis of cellulose is β-glucosidases. Even though strictly speaking these are not cellulases, they are necessary to hydrolyze cellobiose into glucose.

T. reesei cellulases are multi-domain proteins consisting of a Carbohydrate-Binding Module (CBM), a linker chain and a catalytic domain [101–103]. The structure of the catalytic domains has been solved, revealing a tunnel-shaped catalytic domain for CBH I and II [104,105] and an open cleft binding site in EG I

[106]. The structures match the known primary modes of action, i.e. the processive hydrolysis catalyzed by CBH I/II and endo-activity of EG I.

The main function of CBMs (Type A) is to increase the enzyme affinity towards crystalline cellulose [101]. Isolated CBMs [107] and intact cellulases, such as CBH I [108], have shown preferential adsorption to the hydrophobic (110) plane of cellulose microfibrils isolated from micro-algae; however it is not known if CBMs adsorb to the same crystal plane of microfibrils in higher plants [40]. There is evidence for both reversible (CBH I [109]) and irreversible (CBH II [110]) binding of CBMs to crystalline cellulose, indicating differences in affinity. CBMs also contribute to the unspecific binding of cellulases to lignin [111], which can in part explain the irreversible binding of cellulases to lignin-rich biomass, such as spruce [112]. The importance of CBMs for enzyme localization may depend on hydrolysis conditions, as *T. reesei* cellulases with their CBMs removed can achieve the same cellulose conversion as intact enzymes, when the hydrolysis occurs at high solid loading [113]. In addition to enzyme localization, CBMs have been claimed to disrupt crystalline cellulose, however the evidence is scarce [94].

Inhibition of cellulases has been widely studied, and a large number of compounds are listed as possible inhibitors, including glucose, mannose, galactose, xylose, ethanol and various ions [94]. However, the disaccharide cellobiose seems to be a more potent direct inhibitor of cellulases than the previously mentioned compounds [114]. Evidence indicates a higher inhibitory effect of cellobiose on CBHs than EGs, likely due to the closed catalytic domain of cellobiohydrolases [115].

Synergy between cellulases is a known property of cellulase systems. However, the exact nature of the synergistic effects has been difficult to elucidate, due to the complex activity of the individual cellulases and their interactions on a heterogeneous substrate. Initially, EGs were thought to attack amorphous regions of cellulose and expose new chain ends needed for CBHs to initiate hydrolysis. However, observed properties of cellulases and their interactions, such as higher affinity of EG I than CBH I towards crystalline cellulose [116], endo-initiation by CBH I and II [117,118] and observed optimum synergistic ratios [119], have been difficult to reconcile with this model. Recently, the idea of steric hindrance [120] or amorphous cellulose as an obstacle to CBH processivity [121] has been introduced, where cellulase cooperation improves hydrolysis by removing obstacles on the cellulose surface [122,123].

Most recently, the C_1-C_x model has been revived by the discovery of lytic polysaccharide monooxygenases (LPMOs), which are potent enzymes that depolymerize crystalline cellulose through a highly exergonic oxidative mechanism. LPMOs are known to oxidize C1 and/or C4 atoms of a glycosidic bond; however, the details of the mechanism are still under debate [124]. In order

to maintain activity LPMOs require both a reducing agent and molecular oxygen [125]. No external electron donor is needed if hydrolysis is performed on lignocellulose, indicating that lignin or lignin-derived compounds can perform this role [126]. Modern commercial cellulase cocktails are thought to have significant LPMO activity. A schematic representation of cellulose hydrolysis is shown in Figure 2.5.

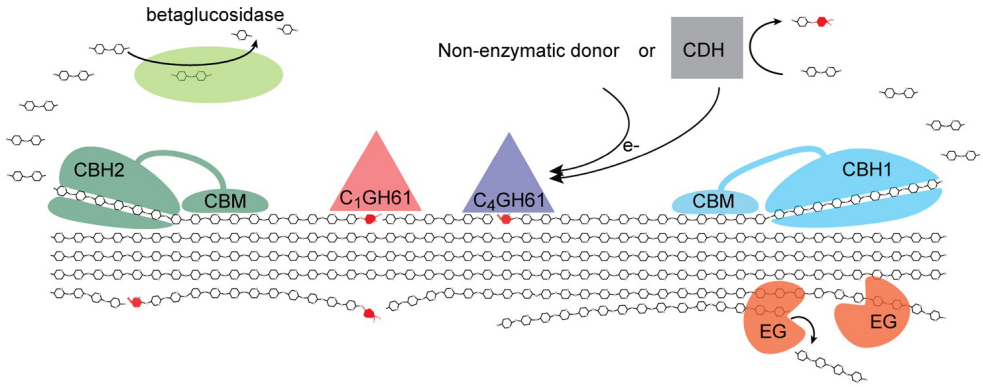

Figure 2.5. Schematic representation of cellulose hydrolysis, including oxidation by LPMOs. Image adapted from [127].

3. Particle size and rheology

Techno-economic studies have indicated that biomass should be processed at high solid loading in order to lower both the capital costs, i.e. cost of equipment (CAPEX) and decrease the operating costs (OPEX) in terms of process energy needs [128–132]. However, mechanical handling, i.e. pumping and mixing of high solid pretreated biomass, is difficult due to the physical properties of the material. The high yield stress of biomass slurries increases the utility costs for pumping [133] and may also cause the formation of reactor stagnant zones when under insufficient mixing [134]. In addition, the high viscosity of pretreated biomass can greatly increase the energy requirements for reactor mixing during enzymatic hydrolysis [135–137].

To address these challenges, it is necessary to characterize and understand the rheology of pretreated biomass. The rheology of large particle suspensions, such as pretreated biomass, is affected by the volume fraction [138,139], size distribution [138,139] and aspect ratio [140,141] of the particles. Accordingly, the rheological study of pretreated biomass should be complemented by the study of the particle/fiber morphology. Moreover, the rheology of pretreated biomass is not static, as it undergoes significant changes during the course of enzymatic hydrolysis. Rheology is influenced by changes in the microstructure (1–1,000 μm), i.e. the particle/fiber size of the pretreated material. However, cellulose hydrolysis directly degrades the microfibrils or the nanostructure (1–1,000 nm) of the biomass, and the connection between the changes in nano- and microstructure is not well understood.

3.1 Particle size of pretreated biomass

Particles encountered in nature are usually polydisperse, i.e. they occur in a wide variety of sizes. Thus, the size of such particles can best be described by a particle size distribution (PSD), i.e. a function that states what fraction of the particles falls within a certain size range. PSDs can be measured by sieving and weighing of the sieved fractions; however, this method requires relatively large samples and is time consuming. Fortunately, commercial instruments for automated

determination of PSD have been developed, based on the measurement of a variety of physical phenomena, such as translational diffusion, rotational diffusion, sedimentation, light diffraction and light scattering [142]. From the PSD it is possible to calculate measures of average particle size, such as the volume mean (d_{43}) and area mean (d_{32}) diameter.

Measurement of light diffraction has become a common method adopted in particle size analyzers, as it requires relatively simple components, such as a monochromatic light source, optical lenses and photodiodes; and as measurements are not performed on individual particles, but simultaneously on a large particle ensemble, it is also rapid and yields reproducible results. However, one disadvantage of commercial instruments, including the one used in this thesis, is the inability to determine particle shape, in part due to the design of the detector [143]. Particles in nature tend to be of an irregular shape; such a shape can be described by an additional measure, such as aspect ratio, which is defined as the ratio of a length and width dimension. Image analysis still remains the preferred method of determining particle aspect ratio.

The first section of Chapter 3 is based on the results of **Paper I**, which discusses the particle size distribution of steam pretreated Norway spruce and giant reed, and its relation to cellulose conversion and viscosity reduction during bioreactor enzymatic hydrolysis. The particle size was measured by laser diffraction analysis with the Mastersizer 2000 (Malvern Instruments, Malvern, UK) particle size analyzer, which allowed for the observation of changes in the microstructure caused by enzymatic hydrolysis or the mechanical action of a reactor impeller.

3.1.1 PSD of steam pretreated spruce and giant reed

The PSD of steam pretreated spruce and giant reed, as reported in **Paper I**, is shown in Figure 3.1, A and B, respectively. Comparing the shapes reveals a noticeable difference, as the size distribution of spruce particles is unimodal, while the peak at 300–700 μm in the particle distribution of giant reed indicates a bimodal distribution. However, the volume mean diameter (d_{43}) for both pretreated materials is similar, being 120.4 μm for spruce and 130.3 μm for giant reed.

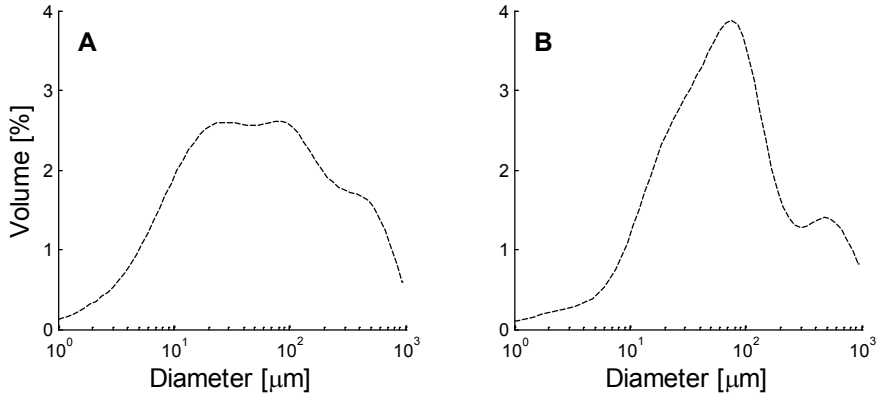

Figure 3.1. The volume based particle size distribution of steam pretreated spruce (A) and giant reed (B), as reported in **Paper I**.

The PSD resulting from continuous steam pretreatment of spruce, as reported in **Paper I**, is somewhat different from previously published results on the particle size of batch steam pretreated spruce [144]. The average particle size of batch steam pretreated spruce was larger, with a d_{43} of 188.3 μm. Milling of batch steam pretreated spruce after pretreatment reduced the average particle size (d_{43}) to 109.0 μm [144]. It is possible that the mechanical action of the screw in the continuous steam pretreatment reactor causes additional reduction in particle size, when compared to batch steam pretreatment. The somewhat bimodal shape of the PSD of steam pretreated giant reed reported in **Paper I**, is likely an indication of high aspect ratio particles or fibers, as these are known to yield an apparent bimodal distribution in laser diffraction measurements [145]. High aspect ratios have been observed in previous studies of the fiber length of pretreated grasses. For example, image analysis of steam pretreated wheat straw showed that high aspect ratio particles (aspect ratio > 4) represented more than 68% of the total fiber length [146].

Overall, these observations suggest that steam pretreatment of grass biomass leads to more fiber separation, creating elongated or high aspect ratio particles of pretreated material. In the case of softwood, the biomass is denser and more lignified, and steam pretreatment does not cause fiber separation to the same extent. Particles of steam pretreated softwood resemble tight fiber bundles, with a somewhat lower aspect ratio; such an appearance is clearly visible in scanning electron microscope images of steam pretreated spruce [147].

3.1.2 Changes in PSD during enzymatic hydrolysis

During enzymatic hydrolysis, one may anticipate changes in both average particle size and particle size distribution. The changes in PSD during enzymatic hydrolysis of steam pretreated spruce and giant reed were also reported in **Paper I**. The materials were hydrolyzed at high solid loading (13% WIS) with a commercial cellulase preparation (Cellic CTec2). The effect of agitation rate was studied by performing the hydrolysis in a bioreactor at different impeller speeds. The changes in PSD during hydrolysis of steam pretreated spruce at 100 and 600 rpm are shown in Figure 3.2, A and B, respectively. Enzymatic hydrolysis by itself was not sufficient to significantly reduce the particle size of steam pretreated spruce, as is evident from experiments at low agitation rate (100 rpm). Particle size reduction at 600 rpm was more significant, indicating that reactor mixing affected the structure of the spruce material. Qualitative observations indicated that the spruce maintained its viscosity during the course of the hydrolysis, possibly due to the inability of the enzymes to cause significant changes in the microstructure of the material.

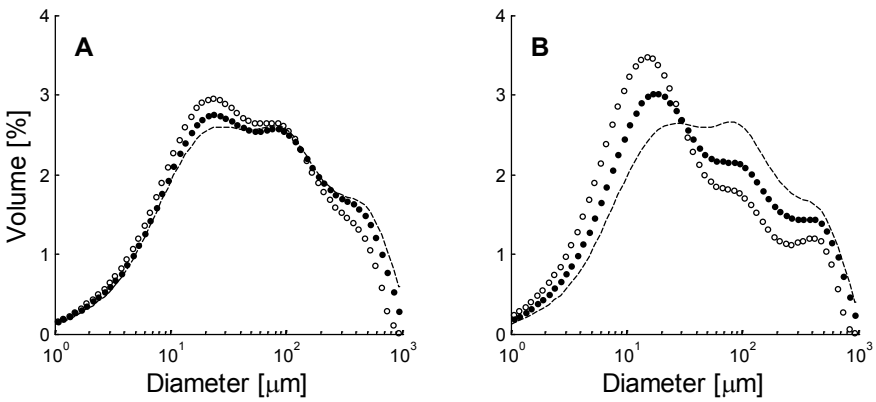

Figure 3.2. The change in volume based particle size distribution of steam pretreated spruce during bioreactor enzymatic hydrolysis at an agitation rate of 100 rpm (A) and 600 rpm (B), as reported in **Paper I**. Dashed line, dot and circle represent the PSD after 0, 24 and 96 hours, respectively.

The changes in PSD during hydrolysis of steam pretreated giant reed at 100 and 300 rpm are shown in Figure 3.3, A and B, respectively. The giant reed material, in contrast to spruce, underwent significant particle size reduction at both 100 and 300 rpm, indicating that the changes in microstructure were primarily driven by enzymatic hydrolysis. Interestingly, the giant reed was also quickly liquefied, transitioning from a soft solid to a low viscosity liquid within the first 8 hours, indicating a link between enzyme driven particle size reduction and decreasing viscosity.

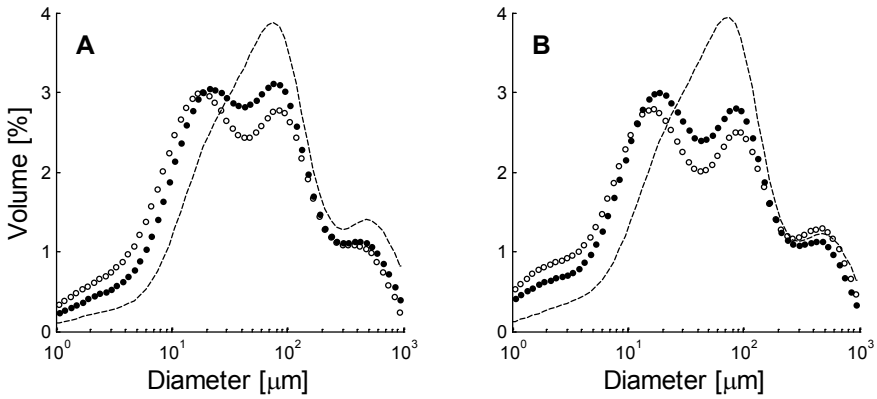

Figure 3.3. The change in volume based particle size distribution of steam pretreated giant reed during bioreactor enzymatic hydrolysis at an agitation rate of 100 rpm (A) and 300 rpm (B), as reported in **Paper I**. Dashed line, dot and circle represent the PSD after 0, 24 and 96 hours, respectively.

3.1.3 The effect of agitation on mean particle diameter

The changes in area mean diameter (d_{32}) during bioreactor mixing and enzymatic hydrolysis of steam pretreated spruce and giant reed are shown in Figure 3.4, A and B, respectively. Comparing spruce and giant reed, the decrease in d_{32} was significantly larger for giant reed than for spruce, which correlates well with the quick viscosity reduction during giant reed hydrolysis. In the case of spruce, the very similar d_{32} profiles for the two experiments at 600 rpm, i.e. (1) 96 hours of hydrolysis and (2) 48 hours of mixing without added enzymes followed by 48 hours of hydrolysis, suggest that the particle size reduction during spruce hydrolysis was primarily driven by intensive agitation.

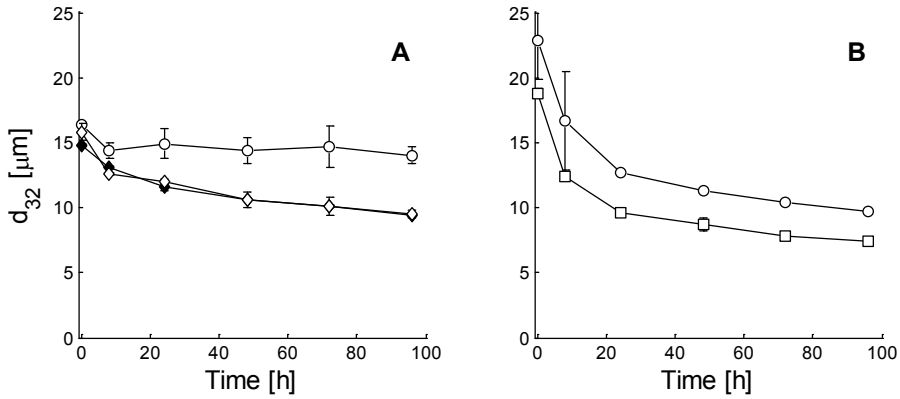

Figure 3.4. The change in area mean particle diameter (d_{32}) of steam pretreated spruce (A) and giant reed (B) during bioreactor mixing experiments as reported in **Paper I**. White symbols represent enzymatic hydrolysis at an agitation rate of 100 rpm (circle), 300 rpm (square), 600 rpm (diamond). In panel A, the black diamonds represent mixing at 600 rpm for 48 hours without added enzymes followed by enzymatic hydrolysis at 600 rpm for 48 hours.

The reduction in d_{32} was more rapid during giant reed hydrolysis. However, this was not due to higher glucan conversion during enzymatic hydrolysis of giant reed. The same glucan conversion levels led to less reduction in d_{32} of spruce when compared to giant reed (Figure 3.5). These results suggest that the extent of particle size reduction does not depend solely on the level of glucan conversion, but also on the type of biomass and pretreatment method.

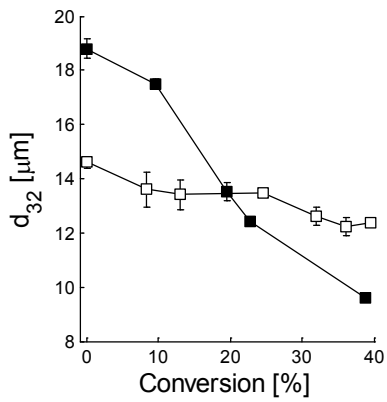

Figure 3.5. The area mean particle diameter (d_{32}) plotted as a function of glucan conversion during bioreactor enzymatic hydrolysis at an agitation rate of 300 rpm, as reported in **Paper I**. White and black squares represent steam pretreated spruce and giant reed, respectively.

The rapid reduction in particle size during hydrolysis of giant reed, as reported in **Paper I**, is in agreement with published reports on hydrolysis of pretreated grasses. For example, in a study on steam pretreated wheat straw, a two-fold reduction in the total fiber length was observed within 6 hours of hydrolysis [146]. The fiber length reduction likely occurs when cellulases "cut" the fibers at dislocations, a mechanism observed in steam pretreated wheat straw [146] and softwood pulp [148]. Mechanical force may be needed to facilitate the breakage of the fibers [149], which may explain the somewhat smaller particle size observed during the hydrolysis of giant reed at higher agitation rate, as reported in **Paper I**.

The effect of agitation on the particle size of steam pretreated spruce has not been previously studied. However, the results from **Paper I** can be compared with other published reports on enzymatic hydrolysis of pretreated softwood. For example, a study on batch steam pretreated spruce reported an initial reduction in particle size (d_{32}) from 24.6 to 16.1 μm within 3 hours, followed by a gradual decrease during the rest of the hydrolysis [144]. However, in this case the effect of agitation on the particle size was not accounted for. Another study reported rapid size reduction during enzymatic hydrolysis of organosolv pretreated lodgepole pine; the average fiber length decreased from 1.72 to 0.54 mm within 3 hours of hydrolysis [150]. A later study that compared batch steam pretreated Douglas fir and organosolv pretreated lodgepole pine, showed that the measured fiber length (not including fines) was significantly longer in organosolv pretreated pine. In addition, fiber length reduction was much more rapid during enzymatic hydrolysis of the organosolv pretreated material [151].

Overall, these observations suggest that pretreatment based on pulping, such as organosolv pretreatment, generates long softwood fibers, whereas continuous steam pretreatment reduces the fiber length, likely by a combination of acid hydrolysis and mechanical action. Fiber length reduction during enzymatic hydrolysis of such "pulped" softwood materials is rapid, and likely caused by enzymatic "cutting" of the fibers at dislocations. Another important difference between the two types of pretreatment is the presence of large amounts of lignin in steam pretreated softwood that prevents significant fiber separation during the pretreatment. Enzymes are not able to rapidly reduce the particle size of steam pretreated softwood, likely due to the lack of fiber separation, and mechanical action from the impeller is needed to break apart the fiber bundles.

3.2 Rheology of pretreated biomass

Rheology can be defined as the study of flow and deformation of matter, and involves concepts such as viscosity, elasticity, plastic and time dependent behavior. Viscosity can be thought of as the internal resistance of fluids to flow. Isaac Newton, in his *Philosophiæ Naturalis Principia Mathematica*, proposed what is today referred to as the Newtonian or constant viscosity model [152]. The Newtonian viscosity, μ (Pa·s), can be defined as the proportionality constant between the shear stress, τ (Pa), and the shear rate, $\dot{\gamma}$ (s^{-1}), for a simple shear flow:

$$\tau = \mu\dot{\gamma} \quad (3.1)$$

The first accurate measurements of the Newtonian viscosity of a fluid were not performed until the late 1830s [153]. In the early 20th century the attention turned towards complex fluids exhibiting non-Newtonian, or shear rate dependent, viscosities, η (Pa·s). E.C. Bingham introduced the concept of the yield stress (τ_y), i.e. the idea that certain fluids do not flow at shear stresses lower than the yield stress [154], and developed the Bingham viscosity model:

$$\tau = \tau_y + \eta\dot{\gamma} \quad (3.2)$$

In the 1920s W. Ostwald and A. de Waele introduced the widely used power law viscosity model:

$$\tau = K\dot{\gamma}^n \quad (3.3)$$

where K and n are usually referred to as the flow consistency and flow behavior index, respectively [155,156]. With this model it was possible to mathematically describe a viscosity that changes with changing shear rate. If the viscosity of a fluid decreases with increasing shear rate, it is defined as a shear thinning fluid, and the flow behavior index, n, falls within the range of 0 to 1.

Several studies on the rheology of pretreated biomass have been published. Most were performed using commercial rotational rheometers; a notable exception is a set of publications on magnetic resonance based rheometry [157,158]. Measuring the rheological properties of dense particle/fiber suspensions, such as pretreated biomass, with a rotational rheometer, poses specific challenges. Pretreated biomass at high solid loading usually has a high and non-Newtonian viscosity, in addition to plastic or yield point behavior. Due to the solid-like properties of the pretreated material it is difficult to insert or contact the measuring system with the sample, and maintain sample homogeneity during the measurements. An additional issue that arises with particle/fiber suspensions is wall slip, a wall depletion phenomenon that decreases shear stress readings, especially at low shear rates [159]. Studies evaluating the utility of different measuring systems and rheological techniques as applied to biomass (pretreated corn stover) have been

performed [160,161]. The results indicate that the serrated plate-plate and the vane-cup measuring systems mostly solved the issues associated with wall slip, and could, if used with care, ensure homogenous behavior of the sample. Additionally, decreasing shear rate ramp experiments were found to be most suitable for steady state viscosity measurements, while oscillatory stress sweeps could provide accurate measurements of yield stress.

The viscosity of high solid pretreated biomass is typically shear thinning, as has been reported for steam pretreated corn stover [162,163], barley straw [164] and spruce [144]. The flow consistency index, K, has a power law dependence on water insoluble solid loading, as reported for different types of pretreated biomass [144,162,163]. Additionally, yield stress values have been measured for different materials, such as corn stover [160] and spruce [144], again showing a power law dependence on solid loading. The strong effect of water insoluble solid loading on viscosity and yield stress is a challenge to effective pumping and mixing of high solid pretreated biomass.

3.2.1 Rheological measurements performed in this study

The remainder of Chapter 3 will discuss the results of **Paper II** and **III**, which describe the changes in viscosity during enzymatic hydrolysis of steam pretreated Norway spruce, batch steam pretreated Scots pine and sulfite pretreated Norway spruce. The effect of agitation rate on the viscosity of steam pretreated spruce was studied during enzymatic hydrolysis in 4 m^3 stirred tank reactors at the Biorefinery Demo Plant (Örnsköldsvik, Sweden) [88], as reported in **Paper III**. Viscosity reduction during hydrolysis of steam pretreated pine and sulfite pretreated spruce, using a complete cellulase system (Cellic CTec3) or a pure endoglucanase preparation (a fungal Cel5A), was studied in **Paper II**.

Two different rheological instruments were used in this work. A rotational rheometer capable of shear rate and shear stress control (Kinexus, Malvern Instruments, Malvern, UK), fitted with a vane and serrated cup measuring system (Figure 3.6), was used to measure viscosity by decreasing shear rate ramp experiments, as reported in **Paper III**. A shear rate controlled rotational viscometer (Visco 88, Malvern Instruments, Malvern, UK), fitted with a vane-smooth cup measuring system, was used for *in situ* viscosity measurements during enzymatic hydrolysis, as reported in **Paper II**. Moreover, in **Paper II** and **III**, torque measurements during mixing in a 3 L laboratory bioreactor ('Hanna', Belach Bioteknik, Stockholm, Sweden) were used to assess changes in viscosity. The torque on the axle, M (N·m), during laminar mixing is determined by the apparent viscosity, η_a (Pa·s), according to:

$$M = \frac{cND^3}{2\pi}\eta_a \quad (3.4)$$

where D (m) is the impeller diameter, N (s^{-1}) is the agitation rate and c is a proportionality constant [165]. For more information on the bioreactors used for mixing during enzymatic hydrolysis, see Chapter 4 of the thesis.

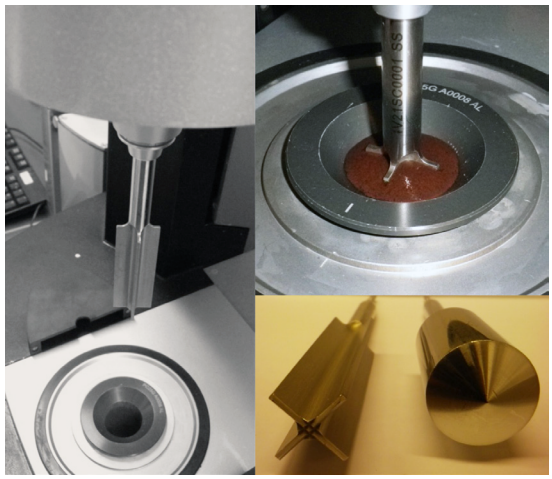

Figure 3.6. The rheometer used for rheological measurements in **Paper I**, **III** and **IV**. The bottom right image shows the common cylinder measuring system (right) and the vane measuring system (left) used for rheological measurements on suspensions.

3.2.2 Initial viscosity dynamics during spruce hydrolysis

From a process perspective, it is highly important to rapidly reduce the viscosity of pretreated biomass. The initial change in viscosity is therefore of particular importance. These initial viscosity dynamics during enzymatic hydrolysis were studied by *in situ* viscometry. Addition of endoglucanases was also tested to determine if it could improve viscosity reduction during hydrolysis of pretreated softwood.

Steam pretreated pine at 12% WIS was hydrolyzed for one hour in a rotational viscometer; the viscosity curves, as reported in **Paper II**, are shown in Figure 3.7. In addition to using a commercial cellulase preparation (Cellic CTec3), a purified Cel5A endoglucanase was also tested, both at a protein loading of 10.4 mg protein per g glucan (mg_{prot}/g_{gluc}). In both cases the viscosity, somewhat counterintuitively, increased during the first 15 min, followed by a gradual decrease for the remainder of the hydrolysis.

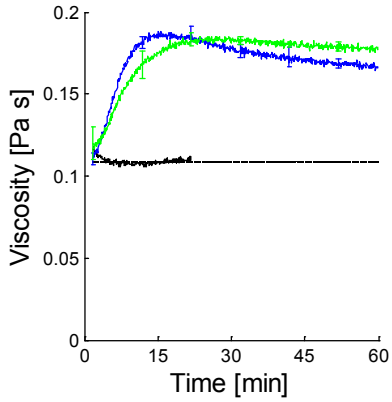

Figure 3.7. *In situ* viscosity at a shear rate of 160.7 s^{-1} during enzymatic hydrolysis of batch steam pretreated pine (12% WIS), as reported in **Paper II**. Black denotes biomass without enzyme addition, while blue and green denotes hydrolysis at a protein loading of 10.4 mg_{prot}/g_{gluc}, with Cellic CTec3 or an endoglucanase preparation, respectively.

In situ viscosity measurements during enzymatic hydrolysis of sulfite pretreated spruce gave qualitatively very different results, as reported in **Paper II**. The spruce material at 2% WIS was hydrolyzed for one hour in a rotational viscometer; the viscosity curves are shown in Figure 3.8. The viscosity was rapidly reduced by Cellic CTec3 and also by the purified Cel5A endoglucanase alone. Even the low endoglucanase dose of 1.04 mg_{prot}/g_{gluc} caused significant viscosity reduction during one hour of hydrolysis.

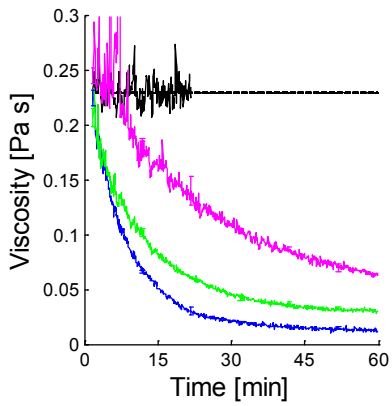

Figure 3.8. *In situ* viscosity at a shear rate of 160.7 s^{-1} during enzymatic hydrolysis of sulfite pretreated spruce (2% WIS), as reported in **Paper II**. Black denotes biomass without enzyme addition, while blue and green denote hydrolysis at a protein loading of 10.4 mg_{prot}/g_{gluc}, of Cellic CTec3 or an endoglucanase preparation, respectively. Magenta denotes endoglucanase hydrolysis at 1.04 mg_{prot}/g_{gluc}.

When comparing the measured viscosity of the two types of pretreated softwood, as reported in **Paper II**, significant differences are apparent. For example, the viscosity of sulfite pretreated spruce at 2% WIS was significantly higher than the viscosity of steam pretreated pine at 12% WIS. These differences were likely caused by the type of pretreatment used. As previously discussed in Chapter 3, pretreatment methods based on pulping are known to produce long, fully separated fibers. Due to lignin removal, the fibers are more porous and occupy a higher volume fraction per unit solid loading. As higher particle volume fraction and aspect ratio increase suspension viscosity [138–141], this is likely a part of the explanation for the comparatively high viscosity of sulfite pretreated spruce.

As reported in **Paper II**, hydrolysis of steam pretreated pine with Cellic CTec3 or the endoglucanase preparation caused an initial increase in viscosity. Similar observations were also made in **Paper III** during *in situ* viscometry of steam pretreated spruce. These were unexpected results, and as far as the author is aware, no such observations from *in situ* viscometry have been previously published. The cellulose conversion levels reached after 15 minutes of hydrolysis are relatively low, especially when using the endoglucanase preparation, so the solid loading and particle volume fraction can be considered constant in these conditions. Instead, it is likely that the increase in viscosity is caused by a shift in the particle size distribution during hydrolysis, as certain changes in the distribution can affect the packing of the particles, thus increasing the viscosity of the suspension [138].

The viscosity of sulfite pretreated spruce was rapidly reduced both by Cellic CTec3 and the single Cel5A endoglucanase, as reported in **Paper II**. The viscosity reduction was probably caused by endoglucanase driven shortening of the fibers, which decreased the fiber aspect ratio and reduced the entanglement of the fibers. Similar reduction in viscosity was previously reported with purified endoglucanases acting on steam pretreated wheat straw [158,166], and was accompanied by reduction in fiber length [158]. Fiber length reduction has also been observed during enzymatic hydrolysis using complete cellulase preparations. For example, the fiber length decreased during enzymatic hydrolysis of wood pulp [148,151]. In the case of wood pulp derived substrates, such as Solka-Floc, a reduction in viscosity has also been reported [157]. Overall, these results suggest that fiber length reduction by endoglucanases is the main mechanism of initial viscosity reduction for fibrous pretreated biomass, such as wood pulp or steam pretreated grasses. In the case of steam pretreated softwood, significant fiber length reduction already takes place during pretreatment, and the addition of endoglucanase protein is unlikely to improve viscosity reduction during enzymatic hydrolysis.

3.2.3 Long term viscosity dynamics during spruce hydrolysis

The effect of mixing on viscosity reduction during enzymatic hydrolysis of steam pretreated spruce was assessed in **Paper III**. Steam pretreated spruce at approx. 13.5% WIS was hydrolyzed in the Biorefinery Demo Plant reactor at different agitation rates; the viscosity of retrieved samples is shown in Figure 3.9. As with the *in situ* viscosity measurements, the viscosity of the material increased initially, but as the hydrolysis proceeded during the significantly longer period of 3 days, the viscosity gradually decreased. The agitation rate had an effect on the viscosity of pretreated spruce, as it was somewhat higher under low intensity mixing, even at the same cellulose conversion level (Figure 3.9 B). As previously discussed in Chapter 3, the particle size of spruce was highly dependent on the mixing intensity, which may affect the viscosity of the material.

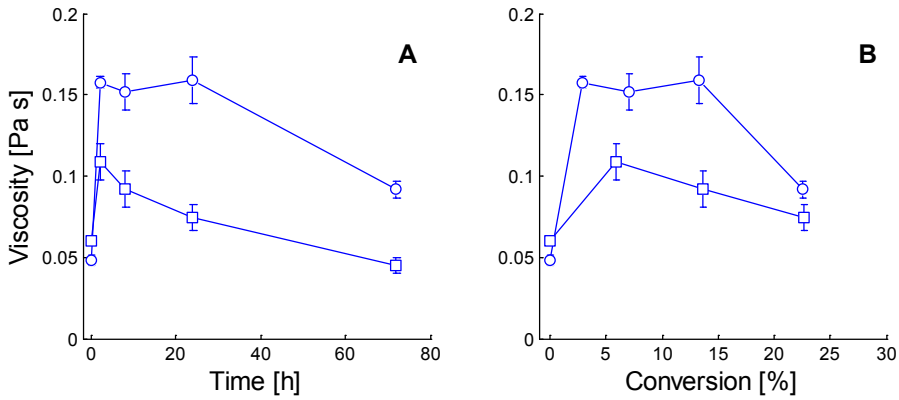

Figure 3.9. Viscosity at a shear rate of 39.8 s^{-1} during enzymatic hydrolysis of steam pretreated spruce (approx. 13.5% WIS) in the Biorefinery Demo Plant reactor (4 m^3), as reported in **Paper III**. Hydrolysis was performed with Cellic CTec2 at a protein loading of 5.4 mg_{prot}/g_{gluc}. Circles and squares represent an agitation rate of 30 and 60 rpm, respectively.

The observed changes in viscosity during hydrolysis of steam pretreated spruce, as reported in **Paper III**, can be compared to the results from a previous study on high solid (10% WIS) batch steam pretreated spruce [144]. The viscosity measured at a shear rate of 32.5 s^{-1} decreased from 0.48 to 0.18 Pa·s during 10 hours of hydrolysis. However, at a shear rate of 10.4 s^{-1}, it increased from 0.84 to 0.92 Pa·s during 3 hours of hydrolysis, followed by a gradual decrease to 0.50 Pa·s after 10 hours of hydrolysis [144]. As previously discussed in Chapter 3, the differences may be related to the differing effect of batch and continuous steam pretreatment on the initial particle size of spruce.

3.2.4 Viscosity reduction with pure endoglucanases

Viscosity reduction was also measured during enzymatic hydrolysis of sulfite pretreated spruce at 6% WIS, as reported in **Paper II**. The material was hydrolyzed in a 3 L laboratory bioreactor ('Hanna') using either a commercial cellulase preparation (Cellic CTec3 at 10.4 mg_{prot}/g_{gluc}) or a very low dose of a purified Cel5A endoglucanase (0.104 mg_{prot}/g_{gluc}). The resulting mixing torque, M (N·m), as measured on the reactor impeller axle, is shown in Figure 3.10. The rapid and non-linear reduction in torque, in relation to elapsed time and cellulose conversion level, is notable. This contrasts to the initial increase in viscosity, followed by a gradual linear decrease, as observed during the hydrolysis of steam pretreated spruce (cf. Figure 3.9).

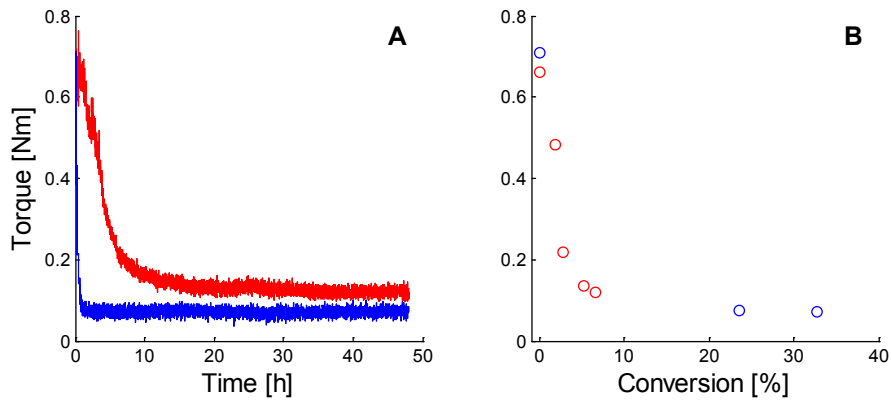

Figure 3.10. Impeller torque for mixing of sulfite pretreated spruce (6% WIS) in the 'Hanna' bioreactor (2 L) at an agitation rate of 50 rpm, as reported in **Paper II**. Blue denotes enzymatic hydrolysis with Cellic CTec3 at 10.4 mg_{prot}/g_{gluc} and red with the endoglucanase preparation at 0.104 mg_{prot}/g_{gluc}.

The rapid reduction in torque during enzymatic hydrolysis of sulfite pretreated spruce, as reported in **Paper II**, is qualitatively similar to the torque decrease observed during hydrolysis of steam pretreated giant reed [137], also a fibrous substrate. Similar rapid reduction in viscosity and yield stress has been observed during hydrolysis of other steam pretreated grasses, such as barley straw [164] and corn stover [163,167]. Again, these results suggest a common mechanism of viscosity and yield stress reduction in fibrous pretreated biomass, such as wood pulp or steam pretreated grasses, by endoglucanase driven fiber length reduction.

A very important conclusion that can be drawn from the endoglucanase driven viscosity reduction in sulfite pretreated spruce, as reported in **Paper II**, is that the viscosity of fibrous substrates can be reduced using much less cellulase protein

(two orders of magnitude less) than what is required to hydrolyze the cellulose in a reasonable time frame. This indicates that it would likely be technically and economically feasible to separate viscosity reduction and cellulose hydrolysis into two process steps in a commercial biorefinery. This may be particularly useful in the context of a "valorization focused" biorefinery, as the pretreatment methods that provide a clean lignin stream usually require washing and dewatering of the cellulose fraction in order to recover lignin and solvents. Fiber length reduction with endoglucanases could in this case be performed on the washed cellulose at low solid loading before dewatering.

4. Mixing and enzymatic hydrolysis

Mixing in a reactor is important for two main reasons: 1) It strongly enhances mass transfer by convective flow, and can affect reaction rates through transport of reactants, products and catalysts; 2) It facilitates heat transfer. However, mixing is associated with an energy cost, since the (normally electric) mixing power is eventually dissipated as heat. Therefore, the effects of increased mixing on process performance need to be understood to properly design the mixing. In this chapter, the specific issue of mixing effects on enzymatic hydrolysis of pretreated biomass is discussed.

The rheology of high solid pretreated biomass, as described in Chapter 3, presents significant challenges to effective reactor mixing. Pretreated fibrous materials at high solid loading, such as agricultural crop residues, usually have rheological properties similar to a soft solid. One practical consideration in enzymatic hydrolysis is to ensure a good initial distribution of enzymes into the solid substrate. This is difficult in a conventional stirred tank bioreactor, prompting researchers to experiment with reactors more suitable for solids mixing. Various horizontal reactor designs, such as rotating paddle reactor [168], scraped surface bioreactor [163] or horizontal rotating bioreactor [169], have been used for liquefaction and hydrolysis of pretreated wheat straw and corn stover. However, horizontal reactors tend to be less efficient for mixing of liquid slurries, and as they operate at large dead-volumes, such designs may increase the capital costs of the overall process.

Vertical stirred tank reactors have been widely used for enzymatic hydrolysis of high solid pretreated biomass, especially for liquid slurries, like steam pretreated softwood [137,170–172]. Fed-batch operation can improve mixing in a stirred tank bioreactor, and has been used for hydrolysis of different types of fibrous biomass, like sugarcane bagasse [136] and wheat straw [173].

Another concern during hydrolysis of biomass, in addition to poor mixing, is high energy expenditure for mixing. Studies have reported mixing power inputs over a wide range of values, depending on different factors, such as reactor geometry, agitation rate, mode of operation, type of biomass and solids content, enzyme loading, etc. Some of the mixing power inputs reported in the literature have been prohibitively high for commercial application (Table 4.1).

Table 4.1. Maximum specific mixing power inputs during enzymatic hydrolysis of pretreated biomass; * - WIS, ** - TS.

	Volume (L)	Biomass	Solids (%)	Power (W/kg)
Scraped surface reactor [163]	8	Corn stover	25*	0.56
Stirred tank reactor [135]	5	Corn stover	30**	38.5
Stirred tank reactor [170]	2.5	Spruce	10*	3.35
Stirred tank reactor [136]	3	Sugarcane bagasse	20**	13.0
Stirred tank reactor [137]	3	Giant reed	20*	1.11

Following the initial distribution of enzymes, it is not known how important mixing or agitation rate is for efficient enzymatic hydrolysis of lignocellulosic biomass. Different studies have reported varying results, e.g. no effect of agitation rate on the hydrolysis of steam pretreated wheat straw [168], a positive effect on the hydrolysis of batch steam pretreated spruce [170] and sugarcane bagasse [174], and even a negative effect on the hydrolysis of cardboard waste [175]. The effect of agitation rate on the enzymatic hydrolysis of steam pretreated spruce has been linked to the relatively slow viscosity reduction during softwood hydrolysis, when compared to grass biomass [137]. However, there is still insufficient understanding of how the type of biomass and pretreatment determine the rheology of pretreated biomass, how the rheology changes during the course of enzymatic hydrolysis, how the changes in rheology in turn affect the quality of the mixing, and how and why the mixing may affect the rate of enzymatic hydrolysis.

4.1 Mixing experiments performed in this study

Contrasting results on the importance of mixing have previously been obtained with different feedstocks. The objective of the current work was to understand why and when mixing will have a strong impact on the enzymatic hydrolysis. Two types of biomass, the softwood Norway spruce and the energy grass Giant reed, were steam pretreated and hydrolyzed with a commercial cellulase preparation (Novozymes Cellic CTec2 or CTec3). The hydrolysis was performed in two laboratory stirred tank bioreactors, a 2.5 L Biostat A+ reactor (Sartorius, Melsungen, Germany) and a 3.0 L 'Hanna' reactor (Belach Bioteknik, Stockholm, Sweden); and a 10 m^3 reactor at the Biorefinery Demo Plant (Örnsköldsvik, Sweden) [88]. All reactors were equipped with one or two pitched blade impellers, while for some experiments in the 'Hanna' reactor a wide anchor impeller was used. The impellers used are shown in Figure 4.1.

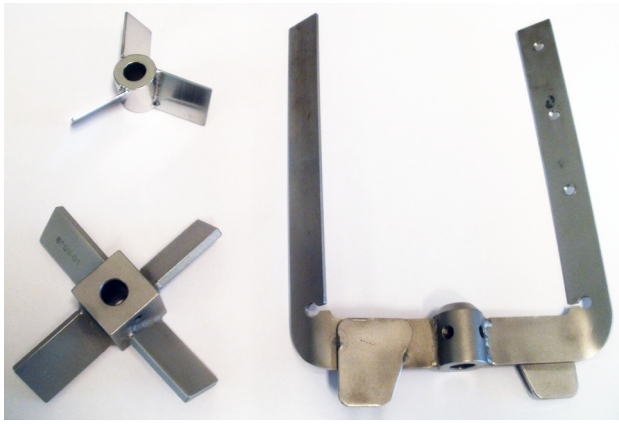

Figure 4.1. The laboratory impellers used in this study. (Left) Pitched blade impellers, (Right) Anchor impeller.

The 'Hanna' reactor was specifically designed with the ability to measure torque, M (N·m), on the impeller axis. Measured torque and agitation rate, N (s^{-1}), was used to calculate the mixing power input, P (W), according to:

$$P = 2\pi MN \quad (4.1)$$

As the viscosity of the pretreated biomass used in this study was shear rate dependent, i.e. shear thinning, an empirical correlation [176] was used to estimate the average shear rate in the reactor, $\dot{\gamma}_{avg}$ (s^{-1}), according to:

$$\dot{\gamma}_{avg} = kN \quad (4.2)$$

The value of the parameter k depends on the impeller design. In this work a value of 11.5 was used for the pitched blade impeller [177] and 20 for the anchor impeller [178]. The viscosity at the average shear rate represented the apparent viscosity in the reactor, η_a (Pa·s). The flow regime in the reactor was characterized by the Reynolds impeller number, Re (-), estimated according to:

$$Re = \frac{\rho N D^2}{\eta_a} \quad (4.3)$$

ρ (kg·m^{-3}) denotes the fluid density and D (m) the diameter of the impeller. The effect of agitation rate, power input or Reynolds number, was evaluated by determining the conversion of glucan during enzymatic hydrolysis. In the case of whole slurry hydrolysis, the glucan conversions presented in Chapter 4 were based on glucose and cellobiose concentrations measured by high performance liquid chromatography, with the exception of results from **Paper III** based on glucose measurements only. The conversion, i.e. fraction of hydrolyzed glucan, C (-), was calculated according to:

$$C = \frac{Glc - Glc_0 + 1.053\ Cb}{1.111\ WIS_0\ x_{G0}} \cdot \frac{1 - WIS_0}{\rho_{l0}} \quad (4.4)$$

where *Glc* and *Cb* are the glucose and cellobiose concentration ($g \cdot L^{-1}$), respectively; ρ_l is the hydrolyzate liquid density in $kg \cdot m^3$; x_G is the glucan mass fraction of the WIS and '0' denotes initial values.

The remainder of Chapter 4 is based on the results of the mixing experiments performed in this study. The first section presents the results of **Paper I**, describing the effect of agitation rate on the enzymatic hydrolysis of steam pretreated Norway spruce and giant reed. The second section details the results of **Paper III**, describing the effects of agitation on enzymatic hydrolysis in the Biorefinery Demo Plant reactor, along with conclusions concerning the importance of flow regime for effective high solid hydrolysis. The final section is based on the results of **Paper IV**, which link the decreased hydrolysis rates to increased end product inhibition due to diffusion-limited mass transfer.

4.2 The effect of agitation rate

The effect of reactor agitation rate on the enzymatic hydrolysis of high solid (13% WIS) steam pretreated spruce and giant reed was investigated in **Paper I**. The glucan conversion during hydrolysis at high (300 rpm) or low (100 rpm) intensity mixing is shown in Figure 4.2. When comparing spruce hydrolysis at low and high solid loading (7 and 13% WIS), it is apparent that higher agitation rate did not improve the hydrolysis over the low solid baseline. However, the low mixing intensity (100 rpm) significantly reduced the hydrolysis rate of spruce at high solid loading, indicating possibly a negative effect of poor mixing. Agitation rate also had a slight initial effect on the hydrolysis of giant reed, possibly linked to the somewhat slower liquefaction of the giant reed at 100 rpm; however, both experiments (100 and 300 rpm) eventually reached similar conversion levels.

The pretreated spruce remained viscous during the course of the hydrolysis, and the outer regions of the reactor volume surrounding the impeller remained stagnant when under low agitation (100 rpm), indicating considerable yield stress in the material. In contrast, the giant reed was liquefied within the first 8 hours of hydrolysis, and following liquefaction, fluid motion was visible in the whole reactor volume, even at low agitation (100 rpm). This indicates that stagnant regions were associated with lower rates of enzymatic hydrolysis.

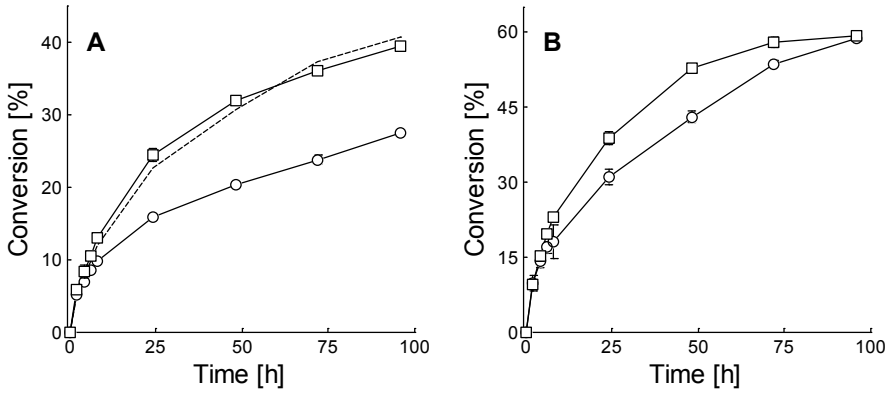

Figure 4.2. Glucan conversion during enzymatic hydrolysis (45 ºC) of steam pretreated spruce (A) and giant reed (B) in the Biostat A+ bioreactor (1 L), as reported in **Paper I**. Circles and squares denote hydrolysis at 13% WIS at an agitation rate of 100 and 300 rpm, respectively. Dashed line denotes low solid hydrolysis (7% WIS at 100 rpm).

Similar to the qualitative observations in **Paper I**, a previous study reported slower viscosity reduction during hydrolysis of steam pretreated spruce, when compared to giant reed [137]. Additionally, impeller speed was previously shown to have an effect on the enzymatic hydrolysis of batch steam pretreated spruce, when performed on 10% WIS at 34 ºC [170]. In contrast, agitation rate had no effect on the hydrolysis of steam pretreated wheat straw [168], i.e. another type of grass biomass. These results indicate that the effect of mixing is linked to properties of the pretreated material, i.e. its rheology and how it changes during enzymatic hydrolysis.

4.3 Reactor scale, mixing power input and flow regime

The effect of mixing on the enzymatic hydrolysis of high solid steam pretreated spruce was also investigated in the Biorefinery Demo Plant reactor. These results were published in **Paper III**. The mixing of the 4 m^3 working volume was performed with pitched blade impellers and the glucan conversion at an agitation rate of 30 and 60 rpm is shown in Figure 4.3. As in laboratory scale hydrolysis, the mixing intensity had a strong effect on the hydrolysis rate.

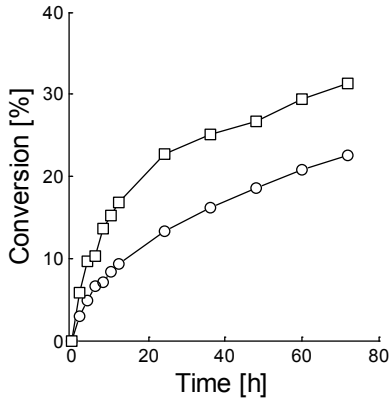

Figure 4.3. Glucan conversion during enzymatic hydrolysis (45 ºC) of steam pretreated spruce in the Biorefinery Demo Plant reactor (4 m^3) at approx. 13.5% WIS, as reported in **Paper III**. Circles and squares denote an agitation rate of 30 and 60 rpm, respectively.

The results of the hydrolysis in the Biorefinery Demo Plant reactor (4 m^3) were also compared to laboratory reactor (2 L) experiments performed with two impeller geometries (pitched blade and anchor) at different agitation rates, for more details see **Paper III**. Torque measurements were used to calculate mixing power input, according to eq. 4.1, and measured viscosities were used to estimate impeller Reynolds number, by means of eq. 3.3, 4.2 and 4.3. The final glucan conversion for all the experimental conditions, as a function of specific mixing power input and Reynolds number, is shown in Figure 4.4. The conversion levels were better correlated with Reynolds number than mixing power input (Pearson's linear correlation coefficient of 0.76 compared to 0.51).

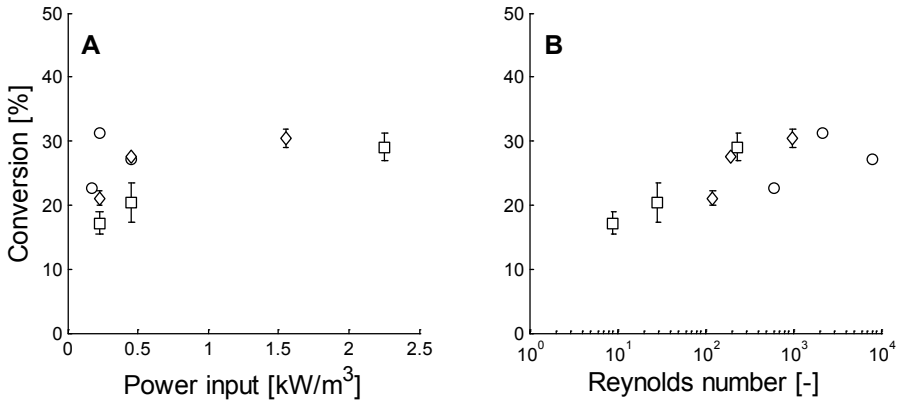

Figure 4.4. Glucan conversion after 72 hours enzymatic hydrolysis (45 ºC) of steam pretreated spruce, plotted against specific power input (A) and initial Reynolds number (B), as reported in **Paper III**. Squares and diamonds represent 'Hanna' bioreactor (2 L) mixing with a pitched blade or anchor impeller, respectively. Circles denote Biorefinery Demo Plant reactor (4 m^3) mixing with a pitched blade impeller.

The positive correlation between glucan conversion and Reynolds number, as reported in **Paper III**, may be linked to the flow properties of concentrated suspensions when under impeller agitation. Mixing studies on yield stress [134] and shear thinning fluids [179] have shown that well mixed caverns or pseudo caverns are formed in the vicinity of the impeller, while the outer regions remain stagnant. As the cavern diameter has a power law dependency on the impeller Reynolds number in the transitional flow regime [134], slower enzymatic hydrolysis in the stagnant regions could in part explain the lower glucan conversion at low Reynolds number, as reported in **Paper III**.

Overall, these results suggest that the specific mixing power input needed for effective enzymatic hydrolysis may decrease with increasing reactor scale, which

is encouraging considering some of the high mixing power inputs measured in laboratory scale experiments (cf. Table 4.1).

4.4 Mass transfer limitations

The idea that formation of stagnant regions negatively affects the cellulose hydrolysis rate was further investigated in **Paper IV**. An experimental problem when comparing high and low solid loading is the fact that both the rheology and substrate concentration change concomitantly, and the effects are thus confounded. In an attempt to decouple the rheology effect from that of high solid loading, a thickening agent (1 wt% Xanthan) was added to low solid (5% WIS) steam pretreated spruce giving an increased viscosity already at a low solid loading. Enzymatic hydrolysis was then performed at different agitation rates (Figure 4.5). Increasing the viscosity increased the effect of agitation on enzymatic hydrolysis, indicating that the effect of mixing was not caused by high solid loading *per se*.

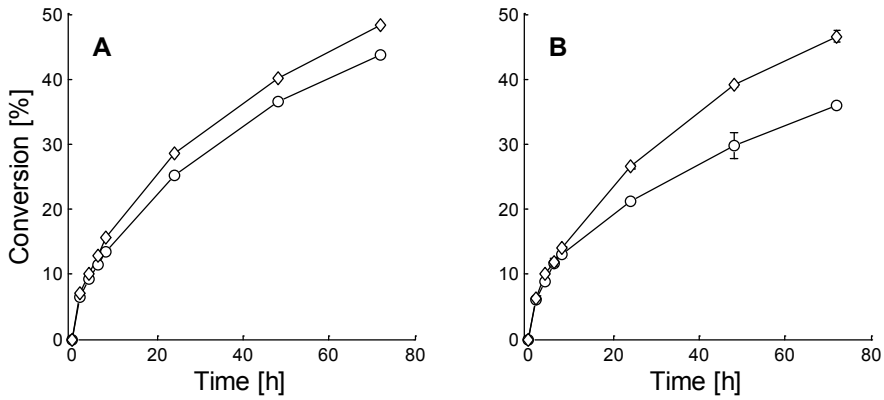

Figure 4.5. Glucan conversion during low solid (5% WIS) enzymatic hydrolysis (34 ºC) of steam pretreated spruce in the Biostat A+ bioreactor (1.2 L), as reported in **Paper IV**. Low viscosity hydrolysis (A) and high viscosity hydrolysis (B), with 1 wt% Xanthan added. Circles and diamonds denote an agitation rate of 100 and 600 rpm, respectively.

A possible explanation for the decrease in enzymatic hydrolysis rate in viscous suspension, when under low intensity agitation, is increased cellobiose inhibition due to mass transfer limitations in the stagnant regions, i.e. a local concentration gradient close to the action site of CBHs and EGs. The mass transfer coefficient in particle suspension is, in part, determined by the slip velocity [180]. The very low

sedimentation velocities of high solid steam pretreated spruce, as reported in **Paper IV**, serve as an indication of the slip velocity in the stagnant regions. Such low slip velocities would cause diffusion limited external mass transfer over the entire range of particle sizes in steam pretreated spruce.

The importance of end product inhibition, when under low intensity mixing, was investigated by continuously removing the formed hydrolysis products by fermentation. Glucan conversion during high solid (16% WIS) simultaneous saccharification and fermentation (SSF) of steam pretreated spruce, as reported in **Paper IV**, is shown in Figure 4.6. The removal of hydrolysis products decreased the effect of agitation, indicating that the effect was indeed caused by increased product inhibition in the stagnant zones.

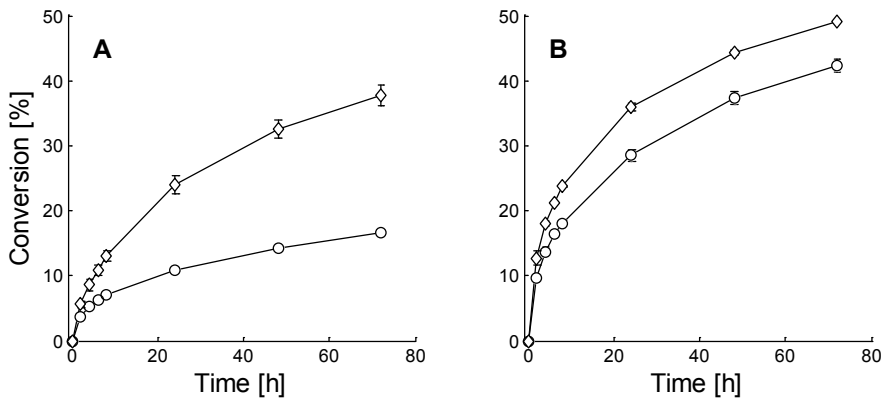

Figure 4.6. Glucan conversion during high solid (16% WIS) enzymatic hydrolysis (34 ºC) of steam pretreated spruce in the Biostat A+ bioreactor (1.2 L), as reported in **Paper IV**. Whole slurry hydrolysis, i.e. high product inhibition case (A) and simultaneous saccharification and fermentation (SSF) of washed spruce, i.e. low product inhibition case (B). Circles and diamonds denote an agitation rate of 100 and 600 rpm, respectively.

The minimal effect of mixing on SSF indicates that it may be a particularly interesting process option for the "energy focused" biorefinery, when processing materials that undergo slow viscosity reduction, such as steam pretreated softwood, as it may significantly reduce the energy cost of mixing.

5. Conclusions

The aim of the thesis was to improve the understanding of the effects of mixing on the enzymatic hydrolysis of pretreated biomass. Additionally, as rheology is an important factor determining the quality of mixing, the changes in rheology during enzymatic hydrolysis, and the connection between rheology and biomass structure, were also studied. New insights on the importance of biomass type and pretreatment in determining the effect of enzymatic hydrolysis on the rheology of pretreated biomass were gained. In addition, a comprehensive explanation of the effects of mixing on the enzymatic hydrolysis of lignocellulose was proposed. The main findings of the thesis are summarized below.

Part I – Particle size and rheology of pretreated biomass

- Particle size reduction during enzymatic hydrolysis of steam pretreated spruce occurs primarily through mechanical comminution caused by the reactor impeller.
- For steam pretreated grasses, such as giant reed, the reduction in particle size is mostly driven by enzymatic hydrolysis.
- For steam pretreated softwood, such as spruce and pine, enzymatic hydrolysis causes an initial increase in viscosity, followed by a gradual decrease during the course of hydrolysis.
- The viscosity of sulfite pretreated softwood – in contrast to steam pretreated softwood – decreases rapidly during enzymatic hydrolysis. Very low doses of pure endoglucanases can reduce the viscosity of such materials without significant cellulose conversion.

Part II – Mixing during enzymatic hydrolysis of lignocellulose

- Agitation has almost no effect on the enzymatic hydrolysis of low solid steam pretreated spruce, likely due to the low viscosity at low solid loading.
- The enzymatic hydrolysis of steam pretreated spruce at high solid loading is strongly influenced by agitation, possibly due to the slow reduction in viscosity during the hydrolysis of steam pretreated softwood.

- The effect of mixing on the enzymatic hydrolysis of steam pretreated spruce remains during scale up to cubic meter size reactors. Importantly, cellulose conversion levels are more determined by flow regime, i.e. Reynolds number, than by specific mixing power input.
- Decreased cellulose conversion during low intensity mixing is likely due to increased local product inhibition caused by mass transfer limitations.
- Agitation has almost no effect on the enzymatic hydrolysis of steam pretreated grasses, such as giant reed, as the viscosity of these materials is rapidly reduced during the initial phase of the hydrolysis.

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