Enzymatic hydrolysis of pretreated barley and wheat straw

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Publication date:
2007

Document Version
Publisher's PDF, also known as Version of record

Citation (APA):
Enzymatic hydrolysis of pretreated barley and wheat straw

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Ph.D. Thesis
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April 2007
Summary.

The work carried out during the Ph. D. project was part of the European research project called the Babilafuente Bioethanol Project and was focused on meeting challenges arising from this project in relation to the enzymatic saccharification of pretreated substrates relevant for the project.

The work involved evaluation of 1) possible ways to increase the glucose release from the commercial cellulase product Celluclast by boosting with other enzyme activities to increase the enzymatic hydrolysis, 2) comparing differently pretreated feedstock substrates and 3) evaluating a fed-batch substrate feeding strategy to increase the substrate loading in the hydrolysis reaction. The substrate for the enzymatic hydrolysis was primarily steam pretreated wheat and barley straw since these substrates were the primary feedstocks for the Babilafuente Bioethanol process.

The initial work showed that there was indeed potential to boost the enzyme activities in Celluclast (arising from *Trichoderma reesei*) by addition of small amounts of fermentation broth from fungal sources other than *T. reesei* at optimal reaction conditions for Celluclast, pH 5, 50 °C. The activity(ies) related to the boosting effect were indicated to arise from more efficient or different endoglucanase activities than those found in Celluclast.

Evaluating of the extent of hydrolysis using the 4 major enzyme activities in Celluclast, which constituted a complete set of enzymes for hydrolysis of cellulose, showed that the most efficient mixture resulted in a glucose release corresponding to ~84 % of the glucose release from Celluclast.

It was therefore suggested that other enzyme activities than the 4 main cellulase activities in Celluclast are necessary for optimal hydrolysis of lignocellulose. Even though Celluclast is a multicomponent cellulase mixture, there are still possibilities for further improvement in terms of providing the most efficient cellulase mixture for lignocellulose hydrolysis.

It was shown that substrates evaluated all had some residual hemicellulose in the solid cellulose fraction after pretreatment. This residual hemicellulose was speculated to be interlocking the cellulose moiety whereas hemicellulolytic activities might benefit the glucose release from cellulase hydrolysis. It is therefore suggested that the boosting effect of enzymes in the fungal fermentation broth might to some extent account for the boosting effect and that the hemicellulolytic activities (and remaining cellulolytic activities...
Summary.

not evaluated) might account for the lower glucose release obtained with monocomponent activities from T. reesei compared to Celluclast.

Evaluation of barley and wheat straw substrates subjected to different pretreatment conditions; hot water extraction and acid- or water impregnation followed by steam explosion showed there were slight differences between the effect of pretreatment conditions in relation to the overall yield from enzymatic hydrolysis. The highest glucose concentration was found for barley straw subjected to acid impregnation followed by steam explosion; however when the glucose concentration was related to the glucose potential in the substrates, the highest yield was obtained with hot water extracted. Analysis of the supernatants from the pretreatments by mass spectrometry showed that the water impregnated straw contained primarily pentose oligomers arising from hemicellulose solubilisation in contrast to the supernatants from acid impregnation.

A substrate fed-batch strategy, that is, sequential addition of substrate or substrate + enzymes during the enzymatic hydrolysis was evaluated in terms of viscosity of the reaction mixture, the glucose release, and overall yield. The fed-batch reactions consistently provided lower concentrations of glucose and yield compared to reaction where all substrate was added at the beginning of the hydrolysis. In terms of glucose release and cellulose conversion it a compromise was necessary to achieve high glucose release and high cellulose conversion. In terms of keeping the viscosity of the substrate slurry at a low level throughout the enzymatic hydrolysis reaction the strategy proved effective; the reactions which were added substrate during the hydrolysis had consistently lower viscosity. The low level of viscosity was thought suggest that mixing of substrate and enzyme would be more efficient.

The work showed that the commercial cellulase product Celluclast can be improved with enzyme activities from other fungal sources and suggested that supplementation of the current multicomponent cellulase product is feasible as a first step to identify promising enzyme activities for lignocellulose hydrolysis. The importance of other enzyme activities other than the main cellulase components was indicated suggesting that increasing the hydrolytic performance could involve addition of hemicellulase activities to complement the cellulase activities found in Celluclast. Further improving the hydrolysis process in relation to the Babilafuente Bioethanol process might be achieved applying a substrate fed-batch strategy, if optimised in relation to timing of the substrate addition, to achieve high substrate loading since this would ensure a low level of viscosity to ensure efficient mixing of substrate and enzymes.
Ph. D. projektet var en del af det Europæiske forskningsprojekt BabilaFuentes Bioethanol projektet og var fokuseret på at imødekomme de udfordringer der opstod i forbindelse med den enzymatiske hydrolyse af forbehandlede substrater relevante for projektet. Arbejdet involverede evaluering af 1) muligheder for forbedring af glukose frigivelsen ved tilsætning af enzym aktiviteter for at "booste" den enzymatiske effektivitet af det kommersielle cellulase produkt Celluclast, 2) sammenligning af forskelligt forbehandlede substrater og 3) evaluering af gradvist tilsætning af substrat til den enzymatiske hydrolyse for at øge substrat indholdet i reaktionsblandingen. Damp eksploderet hvede og byg strå blev primært anvendt som substrater eftersom disse var de mest relevante i forbindelse med BabilaFuentes bioethanol projektet.

I den første del af projektet blev det vist at det var muligt at øge den enzymatiske aktivitet af Celluclast (fermenteringsprodukt af Trichoderma reesei) ved tilsætning af enzym aktiviteter fra fermenteringskulturer fra andre kulturer end T. reesei ved reaktionsbetingelserne pH 5, 50°C der også er optimal for Celluclast. Resultaterne indikerede at de aktiviteter ansvarlige for den øgede enzym aktivitet kunne relateres til mere effektive, eller helt andre, endoglucanase aktiviteter end de fra Celluclast. Ved at evaluere hydrolyse graden opnået med de 4 primære komponenter i Celluclast, hvilke udgør et komplet sæt af enzym aktiviteter for hydrolyse af cellulose, viste at den mest effektive kombination resulterede i en glukose frigivelse på ~84 % af hvad der blev opnået med Celluclast. Disse resultater indikerede at andre aktiviteter udover de fire primære cellulase komponenter er nødvendige for at hydrolysere lignocellulose substratet mest effektivt.

Selvom Celluclast er et multikomponent cellulase produkt så er der stadig muligheder for at forbedre enzym blandingen i forhold til at opnå den højst mulige hydrolyse grad. Eftersom der stadig var noget hemicellulose tilbage i det forbehandlede substrat, kunne det tænkes at den resterende hemicellulose kan blokere cellulosen i det forbehandlede substrat og at enzym blandingen derfor ville kunne blive mere effektiv ved tilsætning af hemicellulose nedbrydende enzymer. Den øgede effekt af tilsætning af enzymer fra fermenteringskulturer andre end Celluclast kan derfor muligvis også forklares ved tilstedeværelsen af hemicellulose nedbrydende enzymer. Den lavere glukose frigivelse opnået ved de 4 monokomponente enzym aktiviteter kan derfor muligvis også tilskrives de øvrige enzymer tilstede i Celluclast.
Sammenfatning.

Sammenligning af byg og hvede halm forbehandlet ved; ekstrahering med varmt vand og syre- eller vand behandling efterfulgt af damp eksplosion, var delvist forskellige i relation til udbyttet fra den enzymatiske hydrolyse. Den højeste glukose frigivelse blev observeret for hydrolyse af syre behandling efterfulgt af damp eksplosion, men når glukose koncentrationen blev relateret til det potentielle glukose indhold i det forbeholdede materiale, så resulterede ekstrahering med varmt vand i det højeste udbytte. Supernatanerne af det forbeholdede strå blev analyseret vha. masse spektrometri. Det blev vist at supernatanerne fra de vandbehandlede substrater indeholdt primært pentose oligomerer fra opløseliggjort hemicellulose, hvilke ikke observeredes i supernatanerne fra de syre behandlede substrater.


I ph. d. projektet blev det vist at det kommercielle cellulase produkt Celluclast kan forbedres vha. tilsætning af enzym aktiviteter fra andre kulturer. Denne strategi virker effektiv som et første skridt på vejen til at identificere nye, effektive enzym aktiviteter til nedbrydning af lignocellulose. Vigtigheden af andre enzymer udover cellulase enzymer blev indikeret og implicerer at tilsætning af hemicellulose nedbrydende enzym aktiviteter vil kunne komplementere de cellulose nedbrydende aktiviteter der allerede findes i Celluclast. For yderligere at forbedre hydrolyse processen i forbindelse med Babilaafuente Bioethanol processen vil gradvis tilsætning af substrat muligvis være en fordel hvis denne strategi kan optimeres mht. til timing af substrat tilsætningen eftersom dette vil resultere i et højt substrat indhold samt lav viskositet af reaktionsblandingen hvorved der opnås en effektiv blanding af enzym og substrat.
Preface.

This project was to a large extent centred around the Bailafuente Bioethanol project.

I would like to thanks to my supervisors Anne and Sven for their help and guidance throughout the project. Anne, your help and guidance has been priceless, very educational, and greatly appreciated.

During the project I have come to know Novozymes people in Bagsværd, Franklinton and Davis with whom I have enjoyed many good discussion and gotten good advice from.

Many thanks to the chemists in Starch R&D especially Anders and Hanne for their help, support, and good advice which they have supplied me with continuously throughout the time I have been working at Novozymes, I look forward to continue working with you.

Thanks also to the lab technicians in Starch R&D for their practical advice and for creating a great working atmosphere in the lab and at lunch, it has been a pleasure.

Til sidst vil jeg gerne takke min familie for deres opbakning og forståelse for al den tid jeg lagt i phd projektet som er gået fra dem og for at give mig rum til at trække vejret og tænke på noget andet efter arbejdstid og min søn Lasse der med smil og latter bringer mig glæde hver dag.

The European Comission is acknowledged for partly financing the work through Framework V programme (contract no. NNE5/2001/685 (The Babilafuent Bioethanol Project)).

Lisa Rosgaard
April 2007.
Preface.
List of publications included in the thesis.

*Biotechnology progress*, 23, 1270-1276.

*Applied Biochemistry and Biotechnology*, 143, 284-296.


*Biotechnology progress*, 22, 493-498.
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1 Introduction.

1.1 Biofuels.

The growing concern for depletion and dependence on fossil fuels has sparked worldwide efforts to replace these with environmentally friendly alternatives and these efforts have been intensified during the last 10 years with the incentives provided by the Kyoto Protocol. Many alternatives to fossil fuels have been suggested; biodiesel, hydrogen, wind energy, solar energy, nuclear fission, and bioethanol, have all been coined as environmentally friendly energy types (Demirbas, 2007). However it is clear that none of these types of energies are capable of providing a perfect substitute for fossil fuels on their own; rather the use of all these technologies as means of providing energy would be necessary for reducing the use of fossil fuels (Pacala and Socolov, 2003; Farrel et al., 2007, Service et al., 2007). The Kyoto Protocol defines biofuels as gas or liquid fuels made from plant material (biomass) or other waste materials: wood, wood waste, wood liquors, wood sludge, spent sulfite liquors, agricultural water, straw, fish oils, tall oil, sludge waste, municipal solid waste, landfill gases and other waste. Bioethanol has attracted much attention for many years and ethanol production from sucrose and starch are already established processes. The major part of bioethanol production is accounted for by the US and Brazil (USDA, 2006).

Ethanol has three principal uses; for production of chemicals, for alcoholic beverages and for transportation fuel. Industrial-grade ethanol is destined for the chemical market (principally, chemical production and medicinal use). Food-grade ethanol (undenatured ethanol), is destined for the alcohol market. Fuel-grade ethanol (bioethanol, or denatured ethanol) is distilled for the energy market for liquid transportation fuel. There are a variety of blending ratios for fuel-ethanol, including E5 (gasoline that contain 5 % ethanol), E10 (10 %), E85 (85 %), and E100 (100 %).

There are basically three types of feedstock for ethanol production:
1. Grain-based; corn, wheat, rice, etc.
2. Non-grain-based; cassava (tapioca), sugar (beets and cane), sweet sorghum, sweet potato.
3. Lignocellulose based; agricultural wastes, grasses, switch grass, plant stalks, trees etc.

The use of lignocellulosic residues has experienced a burst of initiatives world wide to provide an alternative feedstock for bioethanol production (Kim and Dale, 2004; Gray, 2007). Many companies are putting large efforts into creating technology and building
Introduction.

Plants to process lignocellulosic biomass into ethanol (cf. Table 1). Various process variables contribute to the relatively high overall cost of the biomass to ethanol (Hamelinck et al., 2005). Particularly the expense of enzymes utilised in the process has received much attention in terms of creating more efficient products to bring the cost down. To make the whole process of biomass to ethanol feasible it is clear that not only the hexose potential in lignocellulose but also the pentose and lignin fraction must be efficiently utilised. The pentoses in the lignocellulose can be fermented to ethanol or transformed into other products for e.g. xylitol production (Soleimani et al., 2006, Wiedemann et al., 2006). The lignin moiety has several possible applications beside its value as a fuel since lignin might also find use as a bioplastic. In addition, multiple chemicals are considered possible value adding co-products besides ethanol (Kamm and Kamm, 2004; Lasure et al., 2006).
Table 1. Major companies converting lignocellulose into ethanol. The capacity of the plants are listed as million Litres of ethanol per y (ML/Y) or as Tonnes biomass per year (TB/Y).

<table>
<thead>
<tr>
<th>Company</th>
<th>Where</th>
<th>Pretreatment technology</th>
<th>Progress</th>
<th>Capacity</th>
<th>Feedstock</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abengoa Bioenergy</td>
<td>Kansas, USA</td>
<td>Steam, SunOpta technology</td>
<td>Construction start in 2007</td>
<td>329 ML/Y (57 ML from cellulose)</td>
<td>Corn stover, integrated with starch to ethanol process</td>
<td>Hayes, 2007</td>
</tr>
<tr>
<td></td>
<td>Salamanca, Spain</td>
<td>Steam, SunOpta technology</td>
<td>Building plant in Salamanca, Spain, summer of 2007</td>
<td>200 ML/Y (5ML from lignocellulose)</td>
<td>Wheat and barley straw integrated with starch to EtOH process</td>
<td>Abengoa, 2007, Hayes, 2007</td>
</tr>
<tr>
<td>CRAC (COFCO)</td>
<td>ZhaoDong, China</td>
<td>Steam, SunOpta technology</td>
<td>End 2007</td>
<td>6.4 ML/Y</td>
<td>Com stover</td>
<td>SunOpta, Hayes, 2007</td>
</tr>
<tr>
<td>CRAC (COFCO)</td>
<td>Anhui, China</td>
<td>Steam, SunOpta technology</td>
<td>-</td>
<td>440,000 TB/Y</td>
<td>Com stover</td>
<td>SunOpta, Hayes, 2007</td>
</tr>
<tr>
<td>DONG</td>
<td>Denmark</td>
<td>Two-step hot water, counter current extraction</td>
<td>Operational demonstration plant</td>
<td>-</td>
<td>Wheat and barley straw</td>
<td>Dong, 2007</td>
</tr>
<tr>
<td>DuPont</td>
<td>IA, USA</td>
<td>Biorefinery</td>
<td>2007</td>
<td>-</td>
<td>Com stover</td>
<td>DuPont, Hayes, 2007</td>
</tr>
<tr>
<td>Celunol</td>
<td>LA, USA</td>
<td>Steam, SunOpta technology</td>
<td>-</td>
<td>5.3 ML/Y</td>
<td>Sugar cane bagasse, hardwood</td>
<td>Celunol, Hayes, 2007</td>
</tr>
<tr>
<td>Iogen</td>
<td>Canada</td>
<td>Sulfuric acid, steam pretreatment</td>
<td>Operational demonstration plant</td>
<td>4 ML/Y</td>
<td>Wheat, oat, barley straw</td>
<td>Iogen, Hayes, 2003</td>
</tr>
</tbody>
</table>
2 The Babilafuente Bioethanol Project.

The work carried out in the Ph.D. project was done in relation to a European research project called the Babilafuente Bioethanol project, 2001-2005. The project was partly sponsored by the EU under the 5th Framework programme, headed by Abengoa Bioenergy and is among one of the major efforts in Europe to integrate a starch and lignocellulose based ethanol plant in Salamanca, Spain (Table 1). Hence the work carried out in the Ph. D. project was to some extent motivated by the progress and the challenges met in the EU project and were focussed on the enzymatic hydrolysis step (Figure 1).

Initially the work focussed on ways to improve the commercial cellulase product Celluclast specifically by addition of crude fermentation broth cultures presumably containing enzyme activities that could boost the activity of enzymes present in Celluclast. In addition, the effect of monocomponent enzyme activities from Celluclast was evaluated to

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assess the extent of hydrolysis of differently pretreated barley straw substrates. During the project, the effect of different pretreatments and straw feedstocks became interesting since both barley and wheat straw was to be used as feedstocks in the Babilafuente bio-ethanol plant. Hence, we evaluated the enzymatic hydrolysis of barley and wheat straw subjected to different pretreatment conditions. In addition, the effect of a fed-batch substrate loading approach was evaluated to test the hypothesis that gradual addition of substrate would result in lower viscosity throughout the hydrolysis reaction.

The working hypotheses were as follows:

1: The commercial cellulase product Celluclast is widely used for lignocellulose degradation and contains a range of cellulytic enzymes. However, the ratio and the number of isoforms of the cellulytic enzymes in Celluclast might not be optimal or even necessary for hydrolysis of lignocellulose and differently pretreated lignocellulose residues.

2: The hydrolytic efficiency of the commercial cellulase preparation Celluclast might not be optimal for hydrolysis of lignocellulose and could potentially be boosted by cellulytic and/or hemicellulytic enzyme activities from fungal fermentation broth of various fungal sources other than T. reesei.

3: Pretreated lignocellulose is highly water retaining and highly viscous. Since the viscosity is reduced during enzymatic hydrolysis it might therefore be possible to reach high substrate loading by gradually adding batches of substrate. A fed-batch approach would keep the viscosity of the hydrolysis reaction at a low level while retaining a high rate of glucose release and cellulose conversion.

4: Barley and wheat straw require different pretreatment conditions which, in addition to the feedstock straw, will influence the glucose release and degree of cellulose conversion during the subsequent enzymatic hydrolysis.
2.1 Lignocellulosic feedstocks.

Lignocellulosic feedstocks for bioethanol production includes corn stover, grass, straw, softwood, hardwood, rice straw, bagasse, olive tree, etc. (Kim and Dale, 2004, Cara et al., 2006; Peterson, 2006). In either feedstock, the secondary cell wall of the plant material is a dense network of polysaccharides (Figure 2). Cellulose is the main constituent of the plant cell wall and consists of glucose units linked by $\beta-1-4$ glycosidic bonds and is hydrogen bonded to hemicellulose which in is turn ester and ether bonded to the so-called non-core lignin (Bidlack et al., 1992).

Figure 2. Drawing of the components in plant secondary walls. The hemicelluloses moiety is shown in blue and red, cellulose fibrils in orange and the lignin is shown as embedding the cellulose (Modified from Rosgaard et al., 2005).

Hemicellulose constitutes approximately 10-30 % of the typical secondary plant cell wall (Table 2). It is a heterogeneous polymer consisting of D-xylose, L-arabinose, D-galactose and D-mannose with glucoronic, acetic and ferulic acid substitutions and is cross linked to lignin ferulate and diferulate bonds. In grasses, the main part of hemicellulose consists of a xylan backbone with L-arabinofuranose substitutions whereas hardwoods contain also glucomannan with a higher amount of uronic acids (Puls and Schuseil, 1993; Grabber, 2005).
Lignin is an amorphous hydrophobic polymer constituting approximately 9-25% of the secondary plant cell wall in which the cellulose fibrils are embedded. Lignin consists of ferulic acid, diferulic acid, \( \rho \)-hydroxybenzoic acid, \( \rho \)-coumaric acid, sinapic acid and cinnamic acid of which the alcohols of the latter three interact and polymerize into the basic lignin moiety by ether and ester bonds (Bidlack et al., 1992; Grabber, 2005).

The secondary cell wall of plants, trees and grasses differ with respect to the amount of cellulose, hemicellulose and lignin in the cell walls, which in turn also differ within species and with climatic factors (Wiselogel et al., 1996). However, some general comparisons between lignocellulosic feedstocks can be drawn; softwoods contain more lignin than hardwoods, straw has a lower content of cellulose and lignin than wood species and generally contain more hemicellulose than wood. In general, the more lignin present in the native biomass, the lower ethanol yield is eventually obtained (Fan et al., 1982; Vinzant et al., 1997).

Agricultural waste products are considered as a major resource for bioethanol production. E.g. bagasse and corn stover constitute a large amount of biomass which could potentially be used for ethanol; for example, approximately 35% of harvested sugar cane ends up as bagasse after processing and consists of \(~33\%\) cellulose (Table 2) (Martin et al., 2007).


<table>
<thead>
<tr>
<th></th>
<th>Cellulose %</th>
<th>Hemicellulose %</th>
<th>Lignin %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Softwoods (Spruce, Pine, Fir etc.)</td>
<td>41-46</td>
<td>10-13</td>
<td>24-32</td>
</tr>
<tr>
<td>Hardwoods (Aspen, Beech, Poplar etc.)</td>
<td>40-48</td>
<td>19-26</td>
<td>17-32</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>30-38</td>
<td>25-28</td>
<td>16-23</td>
</tr>
<tr>
<td>Barley straw</td>
<td>34-38</td>
<td>19-30</td>
<td>9-15</td>
</tr>
<tr>
<td>Residues from energy crops:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn stover</td>
<td>35-41</td>
<td>16-24</td>
<td>15-17</td>
</tr>
<tr>
<td>Bagasse</td>
<td>33-40</td>
<td>30</td>
<td>15-25</td>
</tr>
</tbody>
</table>

The secondary plant cell wall is to a large extent recalcitrant towards degradation because of the complex polysaccharide and lignin network; however a number of fungi secrete a battery of enzymes such as cellulases, hemicellulases and lignin de-polymerizing enzymes and by the concerted action of these enzymes the native cell wall is degraded in nature.

Several pure cellulose substrates are available for laboratory evaluation of specifically cellulase activity, (Zhang and Lynd 2004; (Zhang et al., 2006). Only short celluoligosaccharides, 1-12 glucose units, are soluble to mildly soluble as is carboxymethyl cellulose
whereas other celluloses are insoluble. Pure cellulose preparations can be obtained commercially or prepared in the laboratory with varying degree of crystallinity; phosphoric acid swelled cellulose is an example of a highly amorphous type of cellulose whereas bacterial cellulose is considered highly crystalline. Specifically filter paper, Whatman No.1, is being used as a means of describing cellulase activity. The standard assaying method published by the National Renewable Energy Laboratory, US (NREL) has been widely applied by many researchers (NREL, 1996; Coward-Kelly et al., 2003). In the present Ph.D. project Whatman No. 1 was applied to assess overall cellulase activity following the recommendations by NREL but also Avicel was applied in some instances to compare cellulase activity on pretreated lignocellulose and pure, insoluble cellulose with intermediate degree of crystallinity.

Pure cellulose substrates provide a relatively simple system for evaluating the effect of cellulose crystallinity and for characterization of the various properties of the individual cellulases. In relation to an industrial process to convert lignocellulosic biomass to ethanol, the model substrates are far from lignocellulose in terms of complexity and accessibility to enzymatic hydrolysis. Since it is imperative that the enzymatic degradation occurs faster than it would otherwise do in nature, the lignocellulosic residues are most often subjected to pretreatment prior to enzymatic hydrolysis.

2.2 Pretreatment.

Most often lignocellulosic biomass is subjected to some sort of pretreatment with the objective of making the lignocellulosic residue more susceptible for enzymatic degradation (McMillan, 1994; Vinzant et al., 1997, Wyman et al., 2005). The factors that affect enzymatic hydrolysis of pretreated lignocellulose are; porosity (i.e. accessible surface area), crystallinity, lignin content, and hemicellulose content which can all be modified by pretreatments (McMillan 1994; Lynd 2002, Laureano-Perez et al., 2005). Pretreatments often involve milling or some kind of size reduction of the biomass residue after which a thermo chemical pretreatment process is initiated. Following this, the lignocellulosic materials will often have an increased surface area due to solubilisation of the hemicellulose moiety, redistribution and/or removal of the lignin compared to the native biomass (Lynd et al., 2002). The impact on removal/redistribution of hemicellulose and lignin depends on the type of pretreatment employed.

Table 3 summarises different pretreatment techniques and show that the optimal pretreatment procedure will depend on the feedstock and on the purpose of the pretreatment is, i.e. if the cellulose moiety is of primary interest, the most effective pretreatment procedure is dilute acid hydrolysis followed by steam explosion, if lignin removal is the
The Babilafuente Bioethanol Project.

primary target then the Organosolv process should be preferred. Other processes to remove lignin are alkaline peroxide treatment which has been shown to increase cellulose hydrolysis and remove up to 80 % of the lignin (Cara et al., 2006). The wet oxidation process has been shown to remove a large part of lignin in various agricultural residues (Schmidt and Thomsen, 1998, Klinke et al., 2002A) and the wet oxidation process is therefore also an attractive pretreatment method for lignocellulosic biomass for ethanol production. Furthermore treatment of wood residues with acetic acid and peroximono- sulphate followed by heat treatment was shown to remove more than 90 % of the lignin originally present in the native biomass; In general removal of lignin and acetate from the biomass greatly improves the glucose release (Kong et al., 1992; Pinto and Kamden, 1996).

If the sole objective is to preserve the hemicellulose moiety and avoid degradation product from the hemicellulose, the hot water extraction procedure is the most efficient (Table 3). The hot water treatment is a relatively mild pretreatment procedure where the hemicellulose is released as oligomers and only a minimal amount of inhibitors are formed. This technology therefore requires a more complex mixture of cellulase and hemicellulase enzymes to fully hydrolyse the biomass after pretreatment to monosaccharides for fermentation (Dien et al., 2006A).

Thus, the criteria for a successful pretreatment procedure include solubilisation of the hemicellulose fraction preferable as oligomers, no or low production of inhibitory compounds, removal of lignin and decreased crystallinity of the cellulose. In the present PhD project acid impregnated, steam exploded straw substrates were primarily evaluated. Steam explosion has been widely applied to many substrates and has been shown to be effective for creating a readily hydrolysable cellulose fraction with almost complete release of hemicellulose as monosaccharides (Table 3). This pretreatment procedure was therefore considered attractive for the Babilafuente Bioethanol process where the initial focus was on hydrolysis of the cellulose moiety.

<table>
<thead>
<tr>
<th>Process technology</th>
<th>Catalyst</th>
<th>Cellulose</th>
<th>Hemicellulose</th>
<th>Lignin</th>
<th>Inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia fiber explosion (AFEX)</td>
<td>Ammonia</td>
<td>Swelling, some de-crystalization, increased surface area</td>
<td>Solubilisation to oligomers</td>
<td>Little effect</td>
<td>Minimal amounts produced</td>
</tr>
<tr>
<td>Alkali treatment</td>
<td>NaOH</td>
<td>Swelling, increased surface area, decrease crystallinity</td>
<td>Partly solubilization</td>
<td>Partial disruption of lignin structure (dependent on feed stock), up to 80% lignin removed</td>
<td></td>
</tr>
<tr>
<td>Dilute acid hydrolysis (0.1-1 % acid) (in conjunction with heat treatment)</td>
<td>H₂SO₄, HCl</td>
<td>Increased pore volume</td>
<td>Almost complete solubilisation</td>
<td>No effect</td>
<td>Several</td>
</tr>
<tr>
<td>Steam explosion (160-260 °C)</td>
<td>SO₂</td>
<td>Increased pore volume, some depolymerization</td>
<td>Almost complete, solubilization</td>
<td>No effect</td>
<td>Several, degradation products from monosaccharides and lignin</td>
</tr>
<tr>
<td>Wet oxidation</td>
<td>Water/alkaline conditions</td>
<td>Some depolymerization</td>
<td>Solubilization to oligomers and monosaccharides</td>
<td>Up to 62 % removal</td>
<td>Minor amounts of degradation compounds</td>
</tr>
<tr>
<td>Over liming</td>
<td>Na₂CO₃, CaOH₂</td>
<td>Some depolymerization</td>
<td>Some solubilization, most is released as oligomers</td>
<td>Removal of inhibitors</td>
<td></td>
</tr>
<tr>
<td>Organosolv</td>
<td>Ethanol, methanol</td>
<td>Some depolymerization</td>
<td>80-100 % removal</td>
<td>Minimal amount of furfural</td>
<td></td>
</tr>
<tr>
<td>Hot water treatment</td>
<td>Water, heat</td>
<td>Some solubilization, most is released as oligomers</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.2.1 Inhibitors.

During pretreatment of lignocellulosic residues various degradation compounds are formed. Some of these inhibit both the yeast but also the enzyme saccharification system, albeit to a lesser extent than the yeast (Gamble et al., 2000, Cantarella et al., 2004). The monosaccharides liberated from hemicellulose during pretreatment are to some extent degraded under acidic conditions at high temperature; acetate is released, furfural is formed from pentoses, 5-hydroxymethylfurfural (5-HMF) is produced from hexoses. Further degradation products include levulinic acid, formic acid and a range of small aromatic molecules from lignin (Klinke et al., 2002A; Chen et al., 2006). Acetate, formic acid and to some extent also levulinic acid has been shown to inhibit enzymatic hydrolysis of steam exploded poplar (Cantarella et al., 2004).

If the pretreated substrate is washed, the inhibitory effect can be avoided (Tengborg, et al., 2001A, Rosgaard et al., 2006 unpublished results). However a washing step is not feasible in an industrial process since large volumes of water would be needed for this and recycling of the process water can only partially recover the volumes of water needed for efficient washing of the pretreated substrate (Stenberg et al., 1998; Wingren et al., 2003). Therefore alternatives to detoxify and otherwise overcome the formation of inhibitors during or after pretreatment have been investigated.

Several methods for detoxification of the pretreated residues have been suggested e.g. by ion exchange using solid resins (Palmqvist and Hahn-Hägerdal, 2000, Weil et al., 2002), evaporation, laccase treatment (Jönsson et al., 1998, Palonen, 2004A), overliming (Martinez et al., 2000), alkali treatment (Persson et al., 2002) or treatment with peroxidase. Overliming of steam pretreated wheat straw has also shown to be very effective in terms of detoxifying pretreated lignocellulose for subsequent hydrolysis and fermentation. The large increase in pH does however cause partial degradation of xylose (Mohagheghi et al., 2006). The phenolic degradation compounds formed during pretreatment can be removed by treatment with laccases or peroxidases which are secreted by many fungi capable of degrading lignocellulosic residues in nature (Thurston, 1994, Call and Mücke, 1997). Jöhnsson et al. (1998) showed that the glucose release of steam pretreated willow increased 3 times following pretreatment with laccase which was correlated to a decrease in the amount of soluble phenolic compounds.

Although several detoxification methods have proven efficient for detoxifying pretreated lignocellulosic residues, it would be preferable if such detoxification steps were not necessary since even a more simple method of detoxification with calcium hydroxide and sodium sulphite has been shown to increase the total cost of ethanol production by ap-
proximately 20 % (von Sivers et al., 1994). In the present Ph. D. project washed pre-
treated substrate was compared to unwashed substrate, and as expected hydrolysis of
washed substrates resulted in a higher glucose release (Rosgaard et al., unpublished
results). However, it was considered that unwashed material would constitute a more
relevant substrate for the Babilafuente Bioethanol project since a washing step should
preferably be avoided in the process, wherefore unwashed pretreated material was pri-
marily evaluated in the Ph.D. work.

2.3 Hydrolysis of cellulose and lignocellulose.

A number of microorganisms are capable of mediating hydrolysis of lignocellulose either
through enzymes associated with the microorganism in cellulosomes or by secretion of
enzymes (Bayer et al., 1996; Lynd et al., 2002). Many fungi produce and secrete various
glycosyl hydrolases (Goedegebuur et al., 2002) and it is well know that these glycosyl
hydrolases act in synergy to efficiently degrade cellulose. These cellulose degrading en-
zymes are grouped in three classes; the exo-acting cellubiohydrolases (EC 3.2.1.91)
which release cellobiose form the ends of the glucose strand, endolucanases which hy-
drolise internal β-1,4-glucoside bonds (EC 3.2.1.4) and β-glucosidase activity (EC
2.1.1.21) which hydrolyse cellubiose to glucose.

2.4 Enzymatic hydrolysis of cellulose.

Trichoderma reesei.

*T. reesei* is one of the most often applied enzyme producing fungi in relation to convert-
ing lignocellulose to ethanol. *T. reesei*, was originally isolated from canvas during World
War II (Reese, 1950, Reese et al., 1976). It secretes 5 different endoglukanases (EG)
(EGI-EGV) or Cel7B, Cel5A, Cel12A, Cel61A and Cel45A, in the nomenclature of gly-
cosyl hydrolase families) and two cellubiohydrolases activities (CBH), CBHI (Cel7A) and
CBHII (Cel6A) (Vinzant et al., 2001). In addition to the CBH and EG activity, *T. reesei*
also produce low levels of β-glucosidase activity (BG); BG1 and BG2 respectively (Chen
et al., 1992). The reason for *T. reesei* to produce isoforms of the cellulases is not entirely
clear. In the present Ph. D. project the commercial cellulase product from *T. reesei*, Cel-
luclast® was used as a benchmark. Although other commercial products and other fun-
gal sources contain cellulose degrading enzymes, Celluclast is considered to be one of
the main commercial products containing high amounts of enzyme protein for efficient
cellulose degradation. In the following sections emphasis is therefore put on understand-
ing the cellulose and hemicellulose degrading enzymes from *T. reesei*. 
Cellulose binding domain.

Most cellulases have a catalytic domain and a cellulose binding domain which are separated by a flexible linker. The cellulose binding domain is found to facilitate binding of the CBH to the cellulose molecule to bring the catalytic site in close proximity with the cellulose (Lynd et al., 2002). The structure of the CBH T. reesei cellulose binding domain (CBD) is a small wedge shaped structure with three tyrosines on the face of the wedge which mediate the binding to cellulose. In the case of EGI, one of the tyrosines is substituted with a tryptophan which is suggested to be responsible for the increased binding affinity of EGI compared to CBHI (Linder and Teeri, 1997). The binding domain is relatively small and covers approximately 10 cellobiose units whereas the catalytic domain of CBHI covers approximately 40-48 cellobiose units (Reinikainen et al., 1995, Sild et al., 1996).

The CBD is important for the hydrolytic effect on insoluble cellulose but has little effect on soluble substrates and has been suggested to 1) mediate cellulose binding for the catalytic module and 2) actively participate in cellulose hydrolysis by displacing individual glucose chains from the cellulose crystal (Rouvinen et al., 1990; Linder and Teeri, 1997). CBHI, II and EGI, II, IV and V, all contain a cellulose binding domain which has been demonstrated to be necessary for efficient hydrolysis for the CBHs but not vital for the hydrolysis as it is with the EGs. However EGIII, which lacks CBD, has a lower affinity for Avicel and phosphoric acid swollen cellulose than the other EGs (Linder and Teeri, 1997; Karlsson et al., 2001). The presence of the binding domain leads to strong association of the entire enzyme with cellulose but the binding affinity varies among the individual enzymes, for example; the CBD from EGI has a higher affinity for cellulose than the CBHI (Linder and Teeri, 1997). The absorption and desorption mechanism is not known and it has been suggested that in some instances the binding is irreversible but considering the processive mechanism and endo activity proposed for the CBHs and EGs (section 2.4.2) the enzymes should be able to move around on the cellulose surface in order to hydrolyse the substrates.

2.4.1 Enzyme composition of T. reesei and structural similarities.

CBHI is the main enzyme produced by T. reesei, followed by CBHII (Table 4). EGI and EGII are the major endoglucanase activities produced (6-20 % of the total cellulase protein secreted). Overall endoglucanases comprise approximately 10-25 % of the total protein found in T. reesei whereas hemicellulolytic enzymes including xylanases, xylosidases etc. comprise less than 10 % (Tolan and Foody) (Table 4). The two CBH activities, CBHI and CBHII have different topology and hydrolyse the cellu-
lose processively from the reducing end and the non-reducing end of the cellulose, respectively, through a tunnel shaped active site. CBHII displays much lower sequence similarity with CBHI than EGI but it does share a region of 36 amino acids well conserved among the EGI, EGII, and CBHI (Teeri et al., 1987; Rouvinen et al., 1990; Divne et al., 1994). EGI share 45% sequence similarity with CBHI and they belong to the same glycosyl hydrolase family (Cel 7) suggesting that these enzymes arose from a common ancestral enzyme; specifically deletions of active site loops present in CBHI account for the difference with EGI. The active site of EGI (and also other endoglucanases) thus presents a groove rather than a tunnel which is capable of hydrolyzing internal bonds in the cellulose chain (Rouvinen et al., 1990; Divne et al., 1994; Henriksson et al., 1996). Interestingly though, CBHI has been shown to have some endo activity and it is suggested that this activity is mediated by transient conformational changes in the loop region creating the tunnel shaped active site to render the tunnel more open and hence able to display some endo activity (Henrissat et al., 1985; Zhang and Lynd, 2004). The EGs also share some structural similarities with xylanases (specifically the GH family 11) although they have little sequence similarity but both EGI and EGII display some xylanase activity as well (Sandgren et al., 2001).
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Table 4. Enzyme components of *T. reesei*. CC= crystalline cellulose, MC microcrystalline cellulose, CMC= carboxymethyl cellulose. * denote calculated values (Vinzant et al., 2001).

<table>
<thead>
<tr>
<th>% of total protein</th>
<th>Mw</th>
<th>Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cel7A (CBHI)</td>
<td>40-60</td>
<td>65 kD, pl 3.8</td>
<td>CBD active on CC, MC</td>
</tr>
<tr>
<td>Cel6A (CBHII)</td>
<td>12-20</td>
<td>58 kDa pl 5.9</td>
<td>CBD active on CC, MC</td>
</tr>
<tr>
<td>Cel7B (EGI)</td>
<td>5-10</td>
<td>55 kDa pl 3.9</td>
<td>CBD, active on MC, CMC, mannan</td>
</tr>
<tr>
<td>Cel5A (EGII)</td>
<td>5-10</td>
<td>44 kDa* pl 4.2</td>
<td>CBD active on MC, CMC, xylan</td>
</tr>
<tr>
<td>Cel12A (EGIII)</td>
<td>1-5</td>
<td>25 kDa pl 5.5-6.8</td>
<td>No CBD active on MC, CMC</td>
</tr>
<tr>
<td>Cel61A (EGIV)</td>
<td>&lt;1</td>
<td>24 kDa pl 5.5*</td>
<td>CBD active on MC, CMC</td>
</tr>
<tr>
<td>Cel45A (EGV)</td>
<td>&lt;5</td>
<td>23 kDa pl 5.1*</td>
<td>CBD active on MC, CMC</td>
</tr>
<tr>
<td>BG1</td>
<td>1-2</td>
<td>78 kDa pl 6.5*</td>
<td>No CBD active on cellulose</td>
</tr>
<tr>
<td>BG2</td>
<td>&lt;1</td>
<td>Not found</td>
<td>No CBD active on cellulose</td>
</tr>
<tr>
<td>Xyn I</td>
<td>N.D.</td>
<td>19 kDa pl 5.2</td>
<td>Xylan</td>
</tr>
<tr>
<td>Xyn II</td>
<td>N.D.</td>
<td>21 kDa pl 9.0</td>
<td>Xylan</td>
</tr>
<tr>
<td>β-xylosidase</td>
<td>100 kDa pl 4.7</td>
<td>β-1,4-xylooligosaccharides</td>
<td>Herrmann et al., 1997</td>
</tr>
</tbody>
</table>

Besides the CBH and EG activity necessary for cellulose degradation, BG activity is also produced (Chen and Esterbauer, 1992; Nieves et al., 1998). However, a large part of the BG enzyme activity is located in the mycelia of the fungus and is therefore not secreted or efficiently recovered during industrial enzyme production (Table 4).

Hence, BG activity is most often applied exogenously by addition of NS 188 to supplement the cellulase product and overcome product inhibition of the CBHs from cellobiose (Messner and Kubicek, 1990; Baker et al., 1998). EG, CBH and BG activities are all subject to product inhibition with cellobiose is the main inhibitor of CBH activity and glucose is the main inhibitor of BG activity (Holtzapple et al., 1990, Philippidis et al., 1993).
2.4.2 Synergy and mechanism of cellulose degradation.

Cellulose hydrolysis is mediated by the synergistic action of endoglucanases and cellobiohydrolases which hydrolyse glucose chains on the surface of the cellulose crystal to soluble cellobiooligomers and subsequent hydrolysis by beta-glucosidase activity to produce glucose (Kadam et al., 2004) (Figure 3). The cellobiohydrolases act in a processive manner to liberate cellobiose from either the non-reducing end or reducing end of the cellulose chain whereas endoglucanases hydrolyse randomly at internal amorphous parts of the cellulose chain (Valjamae et al., 1999) (Figure 3). Therefore the endoglucanases decrease the degree of polymerization of cellulose and reduce the viscosity of soluble cellulose preparations (specifically carboxymethyl cellulose) (Cau and Tan, 2002; Zhang et al., 2006). It has been suggested that water molecules partition underneath the end of a glucose chain while the CBH enzyme is attached to the chain and hereby prevent reanealing/reassociation of the glucose chain to the cellulose fibril. This mechanism facilitates creating accessible chain ends for the CBH to continue hydrolysis to explain the processive hydrolysis of cellulose microfibrils (Himmel et al., 2007).

Figure 3. Schematic representation of hydrolysis of cellulose by cellulases. Cellobiohydrolase activity, e.g. CBHI and II are shown in blue and green. Endoglucanases are shown in red and orange. β-glucosidase activity is shown in pink. Cellobiooligomers, cellobiose, and glucose are indicated on the figure. (Modified from Lynd et al., 2002).
However, it is well known that the concerted action of EG, CBH and BG activities are necessary for efficient degradation of cellulose and it has been suggested that the rate limiting step in cellulose hydrolysis is the solubilisation of the solids cellulose structure to soluble cellobextrins (Nidetzky et al., 1994, Zhang and Lynd 2004, Zhang and Lynd 2006). The specific activities of the endoglucanases differ with respect to the substrate they are applied to. In one study EGI and II were shown to have almost similar activity on Avicel and phosphoric acid swollen cellulose, while EGV displayed the lowest activity compared to other EGs found in T. reesei (Karlsson et al., 2002). EGVIII has slightly lower activity on Avicel and phosphoric acid swollen cellulose than EGI-EGII and has been reported to have relatively broad substrate specificity, including mannanase and xylanase. On carboxymethyl cellulose, the specific activity of EGI was approximately 3 times higher than EGI (Kyriacou et al., 1987; Karlsson et al., 2001). The activity of T. reesei EGIV is shown to have much lower activity than EGI, the main endoglucanase. Therefore it is speculated that EGIV might have some other activity towards a substrate not yet identified although a range of substrates were evaluated in Karlsson et al., 2001.

EGV display higher activity on glucomannan than on β-glucan, which is more pronounced than that of the other EGs (Karlsson et al., 2002). On Avicel, EGV release mainly cellotetraphosphate in contrast to EGI, II and II which produced glucose, cellobiose and to some extent cellotriose. The specific activity of CBHII is approximately twice that of CBHI when measured on Avicel and 1/3 higher activity when applied to amorphous cellulose and filter paper (Tomme et al., 1988; Nidetzky et al., 1994).

Modelling of the four main activities for degradation of cellulose has been done by several authors. Zhang and Lynd (2006) provided a model for hydrolysis of cellulose using the three main components CBHI, CBHII, and EGI since a mixture of these has been shown to act similarly to unfractionated enzyme preparations. It was shown that with an increasing degree of polymerization, the relative activity of CBH decreases which is suggested to be due to the lack of points of attack for the CBH. Likewise, EG activity and synergy between EGI and CBHI decrease as a response to increasing crystallinity of the substrates evaluated (Henrissat, et al., 1985; Zhang and Lynd, 2006). Thus the optimal ratio between EG and CBH activity is dependent on the physical properties of the substrate with the two extremes being highly crystalline and highly amorphous cellulose as well as surface availability. Cellobiohydrolase activity is known to decrease the crystallinity index of cellulose whereas endoglucanase activity increases crystallinity (Kyriacou et al., 1987).

Improvements of CBH and EG activity of T. reesei and other cellulolytic fungi have been studied and evaluated by several groups (Srisodsuk et al., 1997; Zhang et al., 2006). In
one study, addition of an extra catalytic module from *Pichia pastoris* to *T. reesei* EGI resulted in a 4-fold increase in the specific activity compared to the native EGI (Liu et al., 2006).

2.4.3 Hydrolysis of lignocellulose.

One of the major differences between hydrolysis of cellulose and lignocellulose is the presence of the lignin moiety. In pretreated biomass this causes part of the enzymes to be irreversibly bound or precipitated with lignin and the enzymes are therefore not able to continue hydrolysing the cellulose moiety (Sewalt et al., 1997). It has been shown that EGs without CDB (i.e. only the catalytic domains) are still susceptible to binding to lignin (Palonen et al., 2004B; Berlin et al., 2005A). The effect of binding to lignin is therefore not solely related to the cellulose binding domain present on EG I and II. In the case of CBHI it was, however, demonstrated that removal of the CBD reduces the adsorption of CBHI to isolated lignin (Palonen et al., 2004B). Addition of BSA or surfactants such as Tween are known to overcome this effect, supposedly by binding to the lignin moiety and thereby shielding the lignin from the enzymes (Yang and Wyman, 2006, Alkasrawi et al., 2003, Eriksson, et al., 2002A). Addition of such compounds to aid in the enzymatic hydrolysis of lignocellulose is not currently feasible due to cost associated, and it would therefore be preferred if engineered versions of cellulases or naturally occurring cellulases with little or no binding to lignin could be used.

It has been shown that in the initial stages of lignocellulose hydrolysis, the effect of the extent of substrate crystallinity is most pronounced but after extended hydrolysis the amount of lignin present in the sample influences the hydrolysis relatively more (Laureano-Perez, 2005). These results suggest that initially the cellulases are preferentially absorbed to the cellulose moiety but as hydrolysis progresses the proportion of available cellulose decreases, hence the propensity for cellulase to lignin adsorption increase.

The commercial cellulase preparations arising from fermentation of *T. reesei* contain other activities besides the CBH, EG and BG activities which are necessary for complete degradation of the lignocellulose moiety (Kubicek-Prantz et al., 1991, Coughlan et al., 1993; Vinzant et al., 2001). If not all the hemicellulose is removed during pretreatment to produce a solid fraction of cellulose, or a liquid stream of hemicellulose oligomers is produced, hemicellulolytic enzymes must also be applied besides the cellulolytic activities in order to release the total amount of monosaccharides in the substrate. Hemicellulose acting enzymes include endo-1,4-β-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37) which are able to hydrolyse the xylan backbone of hemicellulose. For complete
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Hydrolysis activities necessary for removal of the side group substitutions in hemicellulose are also required. This includes; β-glucoronidases (EC 3.2.1.139), α-galactosidases, α-L-arabinofuranosidase (EC 3.2.1.55), acetyl xylan esterases (EC 3.1.1.72), ferulic acid esterases (EC 3.1.1.73) (Coughlan et al., 1993; Sørensen et al., 2007). The effect of hemicellulolytic enzyme activities was exemplified recently by Berlin et al., 2006. The authors showed that addition of multicomponent enzyme products containing xylanase and pectinase activity greatly enhanced the hydrolysis of lignocellulose.

2.5 Process challenges.

A lot of effort has been put into improving the enzymatic hydrolysis of lignocellulose and to a large extent this has been directed towards reducing the enzyme loading and thereby the cost of cellulase enzymes for the biomass to ethanol process. The National Renewable Energy Laboratory, US, has done extensive work in this area in collaboration with Novozymes and Genencor over a 4 year period. This work resulted in a 30 fold reduction in enzyme cost based on optimising the pretreatment conditions and novel enzyme solutions and is considered a major step forward in realising the biomass to ethanol process (Novozymes, 2005, Ragauska et al., 2006).

Other challenges include the overall process design. Conversion of biomass to ethanol can be done in a two stage process by separate hydrolysis and fermentation (SHF) or, in a one step process by simultaneous saccharification and fermentation (SSF). Also a hybrid process with a pre-saccharification step followed by SSF has been proposed (Stenberg et al., 2000). Each process configuration offers different advantages; in the SHF process the hydrolysis is carried out at a temperature that favours the enzymatic hydrolysis followed by a lowering in temperature to accommodate the yeast fermentation which will release the product inhibition of the enzymes that will occur when glucose and cellubiose builds up as the hydrolysis progress (Wingren et al., 2003). During the SSF process product inhibition is lower since glucose is metabolised almost immediately by the yeast. However, the glucose release occurs at a slower rate since the temperature for efficient fermentation is below the optimum for the cellulases. In the hybrid process, the cellulose is first hydrolysed to some extent in the presaccharification step after which the temperature is lowered, the yeast is added, and as the glucose is being metabolised, the rate of glucose release will continue (Hahn-Hägerdal et al, 2006).

One of the prerequisites for making the biomass to ethanol process economically feasible is to carry out the reaction at high substrate loading to achieve high ethanol yields (Zacchi and Axelsson, 1989; Wingren et al., 2003). However, when the substrate loading is increased, the glucose concentration, and hence the final ethanol concentration, will
increase, but the cellulose conversion decreases suggesting that the substrate or compounds in the substrate inhibits hydrolysis (Tengborg et al., 2001B). Furthermore mixing substrate and enzymes at high substrate loading is difficult and requires special equipment (Mohaghegi et al., 1992; Varga et al., 2004; Jørgensen et al., 2006). One way to obtain high substrate loading would be to gradually add substrate to the reaction i.e. start the hydrolysis at a fairly low substrate loading where the viscosity is relatively high. When the viscosity is reduced due to the enzymatic hydrolysis, more substrate can be added after which the viscosity increase transiently as the cellulases continue to hydrolyse the substrate (Felby et al., 2003). The effect of fed-batch loading of substrate in an SSF reaction has been demonstrated to work successfully to obtain high substrate loading (Öhgren et al., 2006, Rudolf et al., 2005). One of the requirements for the Babilafuente Bioethanol process was high substrate loading in order to reach sufficiently high ethanol concentration. Therefore efforts to increase the substrate loading while still retaining a low viscosity comprised one of the challenges in the present Ph. D. project.

The challenges of realising a viable biomass to ethanol process outlined the past sections motivated the various parts of the present Ph. D. project. These comprised; 1) evaluation of the extent of hydrolysis by the 5 main activities necessary for cellulose hydrolysis in relation to differently pretreated substrates, 2) boosting of Celluclast by addition of enzyme activities present in fungal fermentation broth of various fungal sources other than T. reesei, 3) fed-batch addition of pretreated substrate to the hydrolysis reaction to obtain high substrate loading while retaining the viscosity at a low level, and finally 4) evaluating barley and wheat straw subjected to different pretreatment conditions and comparing these in relation to glucose release and degree of cellulose conversion during enzymatic hydrolysis.
Results and Discussion.
Results and Discussion.

This section describes the results obtained throughout the Ph.D. project and how the individual research projects were motivated. First, pretreated barley and wheat straw was used at low substrate loading. The focus was on evaluating cellulolytic enzyme activities from various fungi in relation to boosting the efficiency of a commercial cellulase product and; secondly on optimising the ratio of monocomponent activities from this product. Further studies were oriented towards increasing the substrate loading to more industrially relevant levels and evaluating the differences in the nature of the feedstock and pretreatment procedure. The results obtained throughout the study are summarised and discussed here and the major findings are presented and concluded upon.

3.1 Evaluating enzymes mixtures for lignocellulose hydrolysis.

Numerous studies have shown that growth of various filamentous fungi on cellulosic substrates results in induction and production of a range of cellulolytic and hemicellulolytic enzymes applicable for hydrolysis of lignocellulose (Reese et al., 1950, Berlin et al., 2005B, Berlin et al., 2006, Gusakov et al., 2006). The commercial cellulase product from Novozymes, Celluclast®, arises from fermentation of the filamentous fungus \textit{T. reesei}. This fungus has been shown to secrete a high amount of enzyme protein capable of hydrolysing various lignocellulose residues. Because Celluclast is already a multicomponent enzyme mixture with high protein content, but not specifically produced with the aim of lignocellulose hydrolysis, we chose the strategy of improving Celluclast for \textit{in vitro} hydrolysis of lignocellulose by boosting the enzyme composition instead of completely replacing it. Therefore fermentation broth cultures from a range of fungi grown on pretreated corn stover were added to Celluclast. These fungi might secrete more efficient cellulases or auxiliary enzymes for efficient lignocellulose hydrolysis which would be revealed as a boosting of the hydrolysis of lignocellulose by Celluclast. Furthermore, we evaluated the extent of hydrolysis by applying purified monocomponent EG and CBH activities from \textit{T. reesei} in relation to hydrolysis of barley straw subjected to three different pretreatment conditions. The different pretreatment conditions were speculated to have different requirements with regard to the spectrum of required enzyme activities for optimal hydrolysis.
Results and Discussion.

3.1.1 Screening for new Fungal Cellulase Systems to boost enzymatic degradation of barley straw lignocellulose.

Assay conditions.
The substrate employed was barley straw subjected to acid impregnation followed by steam explosion and the composition of this substrate was determined by acid hydrolysis followed by high performance anion exchange chromatography to determine the content of monosaccharides. The amount of xylose and arabinose comprised approximately 10% of the dry matter. The cellulose content was calculated from the amount of glucose to comprised approximately 40% of the pretreated straw and the extent of hydrolysis was evaluated as percent of cellulose conversion by relating the glucose concentration obtained in the hydrolysis reactions to the amount of cellulose determined in the substrate.

A total of 11 filamentous fungi grown on pretreated corn stover were evaluated and results relating to 6 of these are presented; these 6 fungi were all shown to be active in hydrolysis of filter paper (Table 5).

Table 5. List of fungal sources from which results are presented, their protein content and activity on filter paper (FPU).

<table>
<thead>
<tr>
<th>Source</th>
<th>Protein concentration (g·L⁻¹)</th>
<th>Activity (FPU·mg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
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<tr>
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</tr>
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</table>

The benchmark enzyme loading (BM) to be improved consisted of 2.3 mg Celluclast 1.5 supplemented with 1 mg Novozymes 188 (ß-glucosidase (NS 188)) per g dry matter (corresponding to 7.5 filter paper units (FPU) and 13 cellobiase units (CBU) per g dry matter). With this enzyme dosage, the glucose release occurred most rapidly within the first 6 hours after which the concentration increased only slightly until 48 hours; therefore assaying conditions for testing the fermentation broth cultures were evaluated after 6 hours reaction time. In addition, the cellobiose concentration was almost zero after 6 hours indicating that the level of NS 188 was sufficient to keep the product inhibition from cellobiose to a minimum. The assay was carried out at 2% w/w dry matter. Compared to what would be economically feasible in relation to an industrial process (Madson et al., 1995), this was a relatively low substrate loading but for the purpose of screening the
Results and Discussion.

effect of fermentation broth cultures, 2 % dry matter provided a slurry which could easily be hydrolyzed in small scale (2 g scale) with minimal requirements for processing equipment. We evaluated various substrate loading conditions and found that when applying the benchmark dosage for 6 hours, the glucose release began to decrease corresponding to a cellulose conversion rate of approximately 75 % at 2 % dry matter (Figure 4) (Rosgaard et al., 2006). A substrate loading of 2 % dry matter was therefore chosen to represent assay conditions where the effect of addition of low amounts of enzyme protein from the fermentation broths would be seen in the later stage of hydrolysis if proven to be effective in boosting Celluclast. Since the fungal sources evaluated were all classified as thermophilic, the supplementation studies were carried out using a surface response design encompassing a temperature range of 35-65 °C (and a pH range of 3.5-6.5) to cover a temperature range within which enzymes from the various fungal sources might perform optimally.

### 3.1.2 Evaluation of the boosting effect of enzymes from fermentation broth cultures on lignocellulose.

The results showed that addition of fungal fermentation broths were able to boost Celluclast and NS 188 notably at pH 5, 50 °C (Figure 5, black stars). In contrast to expected, addition of the fermentation broths did not manifest as a change in the temperature opti-
Results and Discussion.

mum of the fermentation broth and Celluclast enzyme mixture; the optimum found was that of Celluclast; 50 °C. This was thought to relate to the fact that the evaluated fungal strains were initially selected based on their performance at pH 5, 50 °C and that Celluclast constituted the majority of the enzymes applied (10 % of the total protein loading arose from the fungal fermentation broths). Therefore any effect of temperature optimum relating to the high temperature stable enzymes in the fermentation broths might have been clouded by the performance of Celluclast.

The glucose yields obtained for the treatments at pH 6, 40 °C with BM + M. thermophila and BM + C. thermophilum and with the BM + C. thermophilum after the treatments at pH 5, 35 °C; pH 4, 40 °C, and pH 6.5, 50 °C (Figure 5, purple bars with white stars), indicated that the C. thermophilum broth was the least efficient and the most sensitive to pH changes among the broth preparations. P. funiculosum, which is categorized as a mesophile organism, has previously been described to secrete high amounts of cellulases and β-glucosidase (Parr et al., 1983). In our study the P. funiculosum enzymes also seemed to display a pronounced cellulase boosting effect at 50 °C. In contrast, C. thermophilum, known to produce thermostable cellulases (Milner 1977), did not show any significant boosting effect on glucose release when added together with Celluclast and NS 188 (Figure 5, purple bars). These results suggest that the C. thermophilum enzyme broth contained a profile of cellulases similar to those of T. reesei, whereas the broths of the other fungi, which were able to act synergistically with the Celluclast + NS 188 mixture, might contain a profile of cellulases different from that of T. reesei.
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Even the effect of minor addition of enzymes from other fungal sources suggests that there is indeed a potential to boost the hydrolytic performance of Celluclast enzymes. It has since been shown that enzymes from e.g. *Penicillium* species appear more effective than *T. reesei* (Jørgensen et al., 2005; Krogh et al., 2004). On of the “strengths” of *T. reesei* is that it secretes high amounts of enzyme protein, in contrast to the results by Gusakov et al. 2006 where the purified monocomponents of *C. lucknowense* performed significantly better than the crude broth sample was explained by an overall low content of the individual components secreted (Gusakov et al., 2006).

Evaluation of the boosting effect on pure cellulose.

In addition to evaluation of the fungal fermentation cultures on lignocellulose, we also evaluated pure cellulose, using Avicel as substrate to assess whether the nature of the substrate had any effect in relation to the performance of the enzymes present in the fermentation broths. When Avicel was hydrolysed with the benchmark dosage supplemented with fermentation cultures at pH 5, 50 °C there was an increase in the amount of reducing ends compared to reactions with benchmark dosage (Figure 6, white bars) which was not found using pretreated lignocellulose. This increase in amount of reducing ends could not be accounted for by calculation of the amount of reducing ends arising from glucose (Figure 6, dark grey bars) or by the sum of glucose and cellobiose (Figure 6, light grey bars). This suggests that the fermentation cultures contained more active EG activities than Celluclast itself.

![Figure 6. Reducing ends (mol·L⁻¹) produced from Avicel determined by addition of DNS (white bars), equivalents of reducing ends (mol·L⁻¹) calculated from glucose and cellobiose determined by HPAEC (light grey bars), and equivalents of reducing ends (mol·L⁻¹) calculated form the concentration of glucose determined by HPAEC (dark grey bars). Abbreviated names used for the fungal fermentation broths correspond to those used in Table 5. The reaction conditions were pH 5, 50 °C.](image-url)
Results and Discussion.

One of the differences in the nature of these two substrates is that Avicel is a pure form of cellulose whereas the pretreated lignocellulose still contained approximately 10% hemicellulose which might be inhibiting efficient degradation of the lignocellulose structure by Celluclast itself. This could suggest that the enzyme mixture applied for lignocellulose hydrolysis might benefit, not just from cellulase activities in the broths, but also from having xylanase activity. Hence, the boosting effect of the fermentation cultures on lignocellulose might arise from hemicellulolytic enzymes not present in Celluclast, which might be capable of removing the remaining "hemicellulose obstacles" and thereby increase the amount of available cellulose for subsequent hydrolysis. This notion is also supported by the results of Berlin et al. (2006) where it was shown that addition of other activities than cellulases, namely as xylanases and pectinases, increased the overall hydrolytic efficiency of Celluclast.

Since is was shown that Celluclast can be boosted by addition of other fungal sources it became relevant to evaluate if the main activities necessary for cellulose hydrolysis was present in optimal quantities in Celluclast and if these could potentially be optimised. Therefore purified monocomponent enzyme activities from Celluclast were evaluated to asses the efficiency of the minimal cellulase enzyme mixture from Celluclast on lignocellulose.

3.2 Minimal T. reesei cellulase mixture for lignocellulose hydrolysis.

The ratio of enzymes expressed by T. reesei might not reflect what is truly needed for efficient hydrolysis of pretreated lignocellulose and therefore suggests that improvement is possible in relation to optimising the activities in Celluclast. The efforts to produce an artificial mixture of cellulases from various fungal sources to provide a blend of minimal cellulolytic activities more efficient in relation to cellulose hydrolysis than multicomponent cellulase preparations has been evaluated before. However, most of this work has been done using pure cellulose substrates such as bacterial cellulose (Boisset et al., 2001), cotton (Gusakov et al., 2006), filter paper (Nidetzky and Clayesens, 1994, Baker et al., 1998), and Sigmacell (Mansfield and Meder, 2003) and only Gusakov et al. (2006) have dealt with a minimal mixture of cellulases for hydrolysis of lignocellulosic residues. We sought to evaluate the minimal cellulase consisting of CBHI, CBHII, EGII and EGII for hydrolysis of barley straw. The straw substrate had been subjected to hot water extraction, acid, or water impregnation followed by steam explosion. The hydrolysates were carried out at 1% dry matter (w/w) and optimal conditions for Celluclast (i.e pH 5, 50 °C) with
mixtures of the four main activities in Celluclast; CBHI, CBHII, EGI and EGII and compared to the hydrolytic efficiency of Celluclast.

3.2.1 Benchmark; Celluclast + NS 188.

The hydrolysis reactions were carried out in small scale as described in the previous section but at a substrate loading of 1 % w/w dry matter. This was done to avoid effects from substrate inhibition so that the differences in glucose release would arise solely from different combinations of the enzymes and not be affected by the amount of substrate in the reactions. Hydrolysis of the four substrates with a benchmark loading of Celluclast and NS 188 at 2.3 and 1 mg per gram dry matter (w/w) (corresponding to an enzyme protein dosage of 11 and 5 mg protein, respectively) showed that the highest glucose concentration was found in acid impregnated, steam exploded straw, followed by hot water extracted straw and water impregnated, steam exploded straw resulting in the lowest glucose concentration (Figure 7, green, blue and light blue squares). In terms of yield based on the amount of cellulose present in the substrates as determined by acid hydrolysis, the hot water extracted straw had the highest conversion percentage (90 %) followed by the acid impregnated, steam exploded straw. The water impregnated, steam exploded substrate had the lowest cellulose conversion percentage (81 and 78 %, respectively). These results suggest that the cellulose is more readily available in hot water extracted straw which might be related to the severity of this pretreatment process. Hot water extracted straw is marked in green, water impregnated, steam exploded straw (blue) and hot water extracted straw (light blue bars). The corresponding xylose release is shown in pink, red and orange squares, respectively.

![Figure 7. Glucose and xylose release from hydrolysis of pretreated barley straw using 11mg Celluclast and 5 mg NS 188 per gram dry matter. The glucose release from acid impregnated, steam exploded straw is marked in green, water impregnated, steam exploded straw (blue) and hot water extracted straw (light blue bars). The corresponding xylose release is shown in pink, red and orange squares, respectively.](image-url)
Results and Discussion.

Water extraction is less severe and produces less inhibitory compounds than acid treatment and the steam explosion procedure. In turn, the acid impregnated, steam exploded straw might be more readily hydrolysed than the water treated sample since the acid treatment removed most of the hemicellulose and thereby resulted in a more porous substrate without hemicellulose cross linking the cellulose fibrils. Therefore even though the concentration of inhibitory compounds might have been greater in the acid treated sample, this was outweighed by the substrate being more readily hydrolysable. Hence, this substrate resulted in higher cellulose conversion compared to the water impregnated, steam exploded sample.

From the benchmark hydrolysis it was also evident that a higher xylose concentration was found in the hot water extracted sample and the water impregnated, steam exploded sample (Figure 7, orange and red squares), whereas the acid impregnated, steam exploded straw sample resulted in the lowest xylose concentration (Figure 7, pink squares). This is consistent with the effect of acid during pretreatment to efficiently release the hemicellulose (including xylan) to monomers (section 2.2), wherefore only low amounts of xylose was liberated during the enzymatic hydrolysis.

3.2.2 Reconstitution with monocomponent enzymes.

The monocomponent activities CBHI, CBHII, EGI and EGII were added following a mixture design template totalling 56 samples for each substrate. The total protein concentration of the monocomponent enzymes in each sample was 11 mg supplemented with 5 mg protein of NS 188 per g dry matter corresponding to the benchmark protein loading of Celluclast supplemented with NS 188. The results showed that the most pronounced main effects were from EGII and CBHII in the case of the steam exploded substrates; the remaining enzymes displayed no main effects (Table 6). This is in accordance with the cellulose hydrolysis being highly dependent on the synergistic action of CBH and EG activity (section 2.4.2). In line with this; the secondary interaction effects were significant on all substrates with the exception of the interaction effects from EGI×EGII on acid impregnated, steam exploded straw, and EGI×EGII, EGII×CBHII on hot water extracted substrate. The most pronounced secondary interaction effect arose from EGI and CBHI in the case of the two water treated substrates and from EGI and CBHII in the case of acid impregnated straw (Table 6, bold). This might related to the effect of the acid treatment since the acid efficiently releases the hemicellulose moiety and part of the amorphous cellulose with the more crystalline cellulose remaining, whereas the water treatment is considered not as aggressive as the acid treatment. Hence, the higher specificity of
CBHII on more crystalline substrate might account for the more pronounced effect of this enzyme in relation to hydrolysis of the acid treated substrate compared to the water treated substrate which might be considered less crystalline.

Table 6. Main and interaction effects of monocomponent enzymes in the hydrolysis reactions at maximal glucose release. The data are expressed as the percentage each monocomponent enzyme provides of the total, maximal glucose concentration in the hydrolysis with coefficient for each effect, the standard deviation (SD) on coefficients for the main and interaction effects. The model fit ($R^2$) and the standard deviation of the residual error (RMSE) is shown. SE refer to steam explosion.

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Results and Discussion.

3.2.3 Optimal enzyme composition.

From the mixture design experiments the optimal combination of the monocomponent enzymes was determined (Figure 8). In the reaction scheme applied here, the amounts of individual enzymes were varied within a fixed enzyme dosage at the expense of each other. Accordingly, the predicted ratio of enzymes to produce the highest amount of glucose indicated that EGII should be omitted from the mixture since it would otherwise be present at the expense of other enzyme activities. The difference between the optimal

Figure 8. Prediction profiles for the glucose release from acid impregnated, SE treated straw (Acid + SE), water impregnated, SE treatment (Water + SE) and hot water extracted straw (Hot water) as a response to different mixtures of CBHI, CBHII, EGI, and EGII. The predicted values of the optimal ratio of each monocomponent are shown in red. The assay conditions were 6 hours reaction time at pH 5, 50 C at a total protein loading of 11 mg monocomponent activity supplemented with 5 mg NS 188 protein.
enzyme compositions was not very different in relation to the three pretreatments. However the results suggest that the optimal ratios for the hot water extracted and the water impregnated, SE treated straw were more similar compared to acid treated straw; in the water treated substrates more EGI and less CBHI were predicted for the optimal mixture compared to the acid treated straw. This might also be related to the incomplete release of hemicellulose in the water treated samples since EGI has some xylanase activity and might therefore be more useful for water treated straw in order to hydrolyse any remaining hemicellulose which could be cross linking the cellulose moiety.

The optimal ratio of enzyme mixture found here (approximately 25: 50: 25 % of EGI: CBHI: CBHII) does not exactly compare to that found in *T. reesei* where 40- 60 % of the protein is CBHI, 10- 20 % is CBHII and 5-10 % is EGI (c.f. table 4, section 2.4.1). This might be related to the fact that *T. reesei* is not as such produced for lignocellulose hydrolysis therefore the enzyme activities in Celluclast might not be present in an optimal ratio for efficient hydrolysis of pretreated lignocellulose substrates in relation to the bio-ethanol process.

Glucose release.

It was speculated that the amount of BG activity, which was applied exogenously as NS 188, was insufficient in relation to the monocomponents added. Results with the optimal mixture of monocomponent enzyme activities on acid impregnated steam exploded substrate showed that the glucose release did benefit from addition of more BG, almost the same extent as when more BG was added to Celluclast (Figure 9). This would suggest that the lower glucose release observed with the monocomponents compared to Celluclast is not related to insufficient amounts of NS 188 but could be related to some other enzyme activity not present in the mixture but present in Celluclast. It has been reported earlier that addition of xylanase, and other hemicellulolytic components increased the glucose release from various lignocellulosic substrates (Berlin et al., 2005A, Berlin et al., 2006, Gusakov et al., 2006). With the optimal mixture of monocomponent enzyme with 5 mg NS 188, the glucose release is approximately 84 % of that obtained with Celluclast supplemented with 5 mg NS188. The difference in glucose release from the optimal mixture and Celluclast might be related to lack of hemicellulolytic activity in the optimal mixture, and the remaining hemicellulose might interlock with the cellulose. It could also be speculated that the lower glucose release obtained with the optimal mixture was related to the monocomponent, purified form of the enzyme activities having lower activity than the enzyme activities in Celluclast. However, since three of the enzymes were recombi-
Results and Discussion.

nant and purified from host product organisms it would seem unlikely that the activity would be less than that found in Celluclast. It should be noted that at NS 188 addition beyond 25 mg, the positive effect on glucose release was most pronounced for hydrolysis with Celluclast. Therefore it appears that with the ratio of enzymes in Celluclast the best component to add would be BG which is also supported by the results of Berlin et al., 2006 and with results obtained with an experimental cellulase product with high level of BG (Rosgaard et al., unpublished results).

3.2.4 Conclusions

The results obtained with addition of fungal fermentation broth cultures demonstrated that it was possible to boost the activity of Celluclast and NS 188. However, the exact mechanism was not clearly demonstrated. It is suggested that the fermentation broths contained other or more active endoglucanases than Celluclast, as suggested by the higher amount of reducing ends present in the reaction with fermentation cultures added than was the case for Celluclast. It was further indicated that the effect of addition of fermentation cultures appeared to be correlated with the nature of the substrate since there was no difference in the amount of reducing ends found when lignocellulose was used as substrate in contrast to when pure cellulose was used. Since spiking of Celluclast with unknown activities increased the glucose release from lignocellulose then there should be room for improvement of Celluclast. It was shown that the optimal mixture of the main cellulase components, CBHI, CBHII and EGI, did not compare exactly to the actual ratio described for T. reesei within the given cellulase protein dose.
However, since the optimal mix of monocomponents did not result in glucose release higher than that of Celluclast on its own, the results suggest that other activities than the main 4 cellulase activities evaluated here are necessary for efficient degradation of pretreated lignocellulose. This might be a hemicellulolytic activity if the presence of hemicellulolytic activities in Celluclast facilitates degradation of the cellulose moiety by degrading the remaining hemicellulose. The hemicellulose would otherwise be interlocking the cellulose, thus preventing a complete degradation by the cellulases. This is supported by the results of Berlin et al. (2005A) and Gusakov et al. (2006) where it was shown that addition of xylanase and other hemicellulolytic activities increase the glucose release from lignocellulose. Consequently, the effect of boosting Celluclast with fermentation broths from various fungi might also be related to the presence of hemicellulolytic enzymes in the fermentation broths.

In relation to the differences in relation to pretreatment, and notably the use of acid in the pretreatment, indicates that different ratios of the monocomponent cellulases are required for optimal degradation since the optimal mixtures differed to some extent between acid treated and water treated straw. This could be related to the hemicellulose content in the straw samples which was almost completely removed in the acid treated barley straw compared to the water treated barley straw.
3.3 Effects of substrate loading on enzymatic hydrolysis and viscosity of pretreated barley straw.

One of the prerequisites for the efficient utilization of lignocellulose for production of ethanol is to produce a fermentable hydrolysate with a sufficiently high glucose concentration to provide a feasible ethanol concentration for the subsequent distillation. In order to obtain this high glucose concentration a relatively high substrate loading is required. However, due to the high viscosity of most lignocellulosic substrates, it is difficult to operate at solids loadings much higher than approximately 10 % by weight of lignocellulose since this prevents efficient mixing. The high viscosity is mainly due to the presence of the relatively low contents of insoluble materials, but is also a result of the pretreatment process which removes portions of the lignin and hemicellulose and increase porosity to increase the accessibility of the substrate to the cellulas (Thompson et al., 1992). Empirically it is known, that the viscosity of the lignocellulosic substrates decreases as a result of cellulosic activity; presumably because the lignocellulose looses its structure and water binding capacity upon cellulose degradation. A way to increase the solids loading for the enzymatic hydrolysis might be to add multiple batches of substrate to overcome the extremely high initial viscosity which hinders mixing and slows hydrolysis. Furthermore it has been demonstrated earlier that addition of fresh substrate to partly hydrolysed steam pretreated spruce resulted in an increase in glucose release (Eriksson et al., 2002B). This section describes the efforts to study the feasibility of a fed-batch strategy,
that is, sequential loading of substrate or substrate + enzymes during enzymatic hydrolysis, using acid impregnated, steam exploded barley straw following the substrate loading scheme outlined in Figure 10. Following this strategy, to some fed-batch experiments extra enzyme was added corresponding to the final substrate loading of 15 % w/w dry matter at the beginning of the experiment and therefore had a high enzyme to substrate loading the first 0-24 hours before additional substrate was added. Comparable experiments with substrate addition, in conjunction with enzyme, were also evaluated.

3.3.1 Results and discussion.

3.3.2 Assay conditions.

In order to obtain reliable viscosity readings during the fed batch experiment it was necessary to carry out the reactions in sufficiently high working volume. Therefore, the experiments were carried out in 2 kg scale i.e. the fed-batch reactions started in 400 g scale but as the substrate loading was increased, so was the reaction volume since the acid impregnated, SE treated substrate had a dry matter content of 20 % by weight. The reaction volume therefore increased markedly in the fed batch reactions.
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3.3.3 Glucose release.

The reaction with the lowest substrate loading, 5 % total solids (TS) loaded at the beginning of the reaction, had the highest conversion percentage, followed by the 15 % TS reaction and the 10 % reaction (Figure 11A). This was expected since it has been shown previously that hydrolysis of lignocellulose increases with decreasing substrate loading (Tengborg et al., 2001B). The hydrolysis occurred faster in the reactions with lower solids loading but after 48 hours the glucose yields of the 15 % reaction was comparable to the experiments with 5 and 10 % substrate loading.

The fed-batch reactions which had the highest enzyme to substrate ratio in the beginning of the reaction had a markedly higher rate of glucose release compared to the other reactions (Figure 11B). When more substrate was added the glucose concentration was, however, in effect diluted by the large volume of water that was added in conjunction with the dry matter since the substrate contained only 20 % dry matter. The highest final glucose concentration after 72 hours was obtained for the reaction loaded with 15 % dry matter at the beginning of the hydrolysis followed by the reactions starting at 10 % dry matter and added substrate to 15 %. In relation to cellulose conversion or yield, the highest overall yield was obtained in the experiments where all substrate was added at the beginning of the reaction and lowest in the fed-batch reactions (Figure 11A and B).

Therefore, since the glucose concentration obtained initially due to the higher enzyme to substrate ratio, was in effect diluted when more substrate was added and there ap-
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peared to be no net benefit of sequentially adding substrate to increase the total solids loading. This same trend also occurred for the experiments with extra substrate in conjunction with extra enzyme to keep the enzyme to total solids loading constant (Figure 11B). When increasing the substrate loading from 5 % to 10 % while adding extra enzyme, the yield remained fairly high, however, when the dry matter was further increased to 15 %, the glucose concentration was diluted and the conversion of added substrate was not sufficient to reach similar yield as the reaction which was kept at a constant substrate loading of 15 %.

Since the glucose concentration in the fed-batch reactions did not reach comparable levels as it did when all substrate was added at the beginning of the experiment, it is suggested that the shorter reaction time that the freshly added substrate in effect had with the enzyme present in the reaction mixture was insufficient. It might also relate to the lag time needed to efficiently mix in the fresh substrate with the enzyme and substrate already present. The results suggested that if the fed-batch reactions were left to react for longer time then the glucose release might increase to levels comparable to those with all substrate added at the beginning of the experiment.

Since a high glucose concentration desired in relation to efficient ethanol production in the subsequent fermentation step, did not appear to correlate with high cellulose conversion, a compromise between glucose concentration and cellulose conversion can be suggested. For this substrate, the compromise was found to be approximately 12 % total solids (Figure 12).

Figure 12. Glucose release (open squares) and yield (closed squares) from 72 hours hydrolysis of acid impregnated, SE treated barley straw as a response to different substrate loading. The graphs intersect at 12.5 % total solids by weight. The hydrolysis was carried out at pH 5, 50 °C.
Results and Discussion.

3.3.4 Quantification of viscosity.

It was not possible to measure the viscosity of reaction with 10 and 15 % total solids at the beginning of the reaction; the mixtures were simply too dense to be measured within the experimental set up. After 6 hours they did become flow able (Figure 13A). The final viscosity of all reactions loaded with the all substrate added at the beginning of the reaction was approximately similar after 72 hours hydrolysis (from 20 to 48 mPa·sec⁻¹). It was shown that the experiments which had a higher enzyme to substrate ratio in the beginning of the reaction also displayed a markedly faster decrease in viscosity to accompany the rapid glucose release observed (Figure 13B). When more substrate was added, the viscosity increased for a while, but decreased within 24 hours to levels comparable to those were all substrate was added at the beginning of the reaction (42-85 mPa·sec⁻¹, Figure 13B). Likewise it was shown that the reactions which were added extra enzyme together with more substrate, also retained a low level of viscosity throughout the experiment suggesting that there might be a threshold in relation to the amount of total solids, i.e. if the total solids content is gradually increased from 5 to 15 %, then the viscosity can be kept at a low level at all time (Figure 13B).
3.3.5 Conclusions.

In contrast to expectations it was shown that in relation to glucose concentration and cellulose conversion there appeared to be no benefit of gradually increasing the substrate loading. On the contrary for the same total reaction time of 72 hours, it seemed that the extra added substrate is not efficiently hydrolysed in comparison with the reactions where all substrate was added at the beginning of the reaction. This might be attributed to the high content of water which was added with the substrate and to the shorter reaction time the freshly added substrate had with the enzymes in the reaction mixture. Compared to results by Varga et al. 2004 where it was also observed that the hydrolysis reaction decrease in fed-batch reaction at higher substrate loading, this was attributed to insufficient mixing but also to increasing amount of inhibitory compounds in the pretreated substrates. It could be speculated that if the substrate applied in this work had been washed, the glucose release might have continued to increase when more substrate was added. In relation to viscosity it was possible to keep the viscosity at a low level as expected. This implies that the amount of power necessary to mix the substrate in industrial scale would be lower in fed batch reactions than in reactions where all substrate was added at the beginning of the reaction. Since the highest glucose concentration was found in reaction with high substrate loading, but high cellulose conversion is obtained with low substrate loading, a compromise between these two parameters was found at 12 % dry matter.

3.4 Comparison of different pretreatment strategies for degradation of wheat and barley straw.

One of the first prerequisites for the utilization of lignocellulose for production of ethanol is to efficiently convert the lignocellulosic residue to monosaccharides for the subsequent fermentation reaction. To increase the accessibility of the cellulose to enzymatic attack the lignocellulosic substrates have to undergo a physico-chemical pretreatment prior to the enzymatic hydrolysis step (Mosier et al., 2005). In brief, pretreatment may involve a mechanical step for reducing the size of the lignocellulose followed by one or more steps of heating and wetting in the presence of a catalyst. In the case of acid catalyzed pretreatment, the hemicellulose moiety of the lignocellulose is efficiently solubilised, leaving the solid fraction consisting mainly of cellulose and lignin. In cases where no catalyst is used the hemicellulose is thought to be solubilised into polymers by the organic acids present in native lignocellulose (acetic acid and water itself) (Allen et al., 2001; Dien et al., 2005; Dien et al., 2006B).
Results and Discussion.

Steam pretreatment in conjunction with an acid catalyst is known to release the hemicellulose constituents of lignocellulose as monosaccharides. However during the heat treatment some of the released monosaccharides may be degraded to compounds inhibitory to both cellulase enzymes and the yeast during the subsequent fermentation step (Palmqvist and Hahn-Hägerdal, 2000; Linde et al., 2006); washing of the pretreated substrate increase the glucose release (Rosgaard et al., unpublished results). Since a washing step is not feasible in relation to an industrial scale process, only unwashed material was evaluated here to represent the most likely type of substrate which would be employed in the Babilafuente bioethanol project. Hot water extraction is particularly attractive compared to acid treatment and steam explosion since this pretreatment involves no handling of harsh chemicals, and is reported to produce a liquid stream which, in contrast to acid hydrolyzed steam explosion treated lignocellulose, does not inhibit the yeast during the fermentation step since the hemicellulose is released as oligomers and are protected from degradation (Allen et al., 2001). Evidently, the specific pretreatment process best suited to a specific process will depend on a number of factors, including the origin of the lignocellulosic biomass, i.e. whether originating from softwood, hardwood, herbaceous energy crop or other agricultural residues, the amount and nature of inhibitory compounds and whether the C5 monosaccharides are supposed to be utilized (Dien et al., 2005; Palmqvist and Hahn-Hägerdal, 2000; Garrote et al., 1999) In this work we sought to investigate the effect of three principally different pretreatment strategies, where the structure of the straw subjected to steam explosion was much more degraded than the straw subjected to hot water extraction, on subsequent enzymatic hydrolysis of wheat and barley straw biomass by Celluclast supplemented with Novozyme 188.
3.4.1 Results and discussion.

3.4.2 Glucose potential and oligomeric content of the pretreated substrates.

The glucose potential of barley straw was slightly higher than that of wheat straw and the hemicellulose content was slightly higher in the hot water extracted substrates compared to the SE treated substrates. The supernatants from both acid impregnated, steam exploded barley and wheat straw substrates, (Barley 1 and Wheat 1, respectively), mainly contained hexose oligomers from 5 C₆ (HO5) and up to 16 C₆ (HO16) in size (Figure 14, A and C). In addition, the Barley 1 and Wheat 1 supernatants also harbored a few short, pentose oligomers (PO5- PO8, Figure 14, A and C). The water impregnated supernatants from water impregnated, steam exploded barley and wheat (Barley 2 and Wheat 2, respectively), both contained a relatively broad profile of pentose oligomers (Figure 14, B and D). These short pentose oligomers were found in both supernatants both with and without acetic and ferulic acid substitutions.

The water impregnated, steam exploded barley supernatant, Barley 2, moreover appeared to contain some hexose oligomers (from HO5- HO14, Figure 14, B) not found in the water impregnated supernatant from wheat straw (Wheat 2) where only pentose oligomers were found. The MALDI TOF (Matrix Assisted Laser Desorption/Ionisation Time Of Flight) mass spectra thus revealed significant differences of the impact of the acid vs. water impregnation on the oligomer profiles of differently pretreated barley and wheat straw. These differences between acid versus water impregnation can be related to the hemicellulose being almost completely solubilised to monomers (not detected in the MS analysis) in the acid impregnated samples, as where the hemicellulose is released as oligomers during the water impregnated samples.
Results and Discussion.

Figure 14. MALDI TOF MS spectra (sodium adducts) of the liquid fraction from acid impregnated, SE treated barley straw, Barley 1 (A); water impregnated, SE treated barley straw, Barley 2 (B). HO denote hexose oligomer, PO= pentose oligomer, FeA denote ferulic acid and Ac=acetic acid. Numbers refer to the number of monosaccharides in the oligomer.
Results and Discussion.

Figure 14. MALDI TOF MS spectra (sodium adducts) of the liquid fraction from acid impregnated, SE treated wheat straw, Wheat 1 (C); water impregnated, SE treated wheat straw, Wheat 2 (D). HO denote hexose oligomer, PO= pentose oligomer, FeA denote ferulic acid and Ac=acetic acid. Numbers refer to the number of monosaccharides in the oligomer.
Results and Discussion.

It was further shown that in the liquid fraction from the pretreated substrates, the xylose concentration was highest in the acid impregnated, steam exploded samples compared to the water impregnated, and steam exploded samples. However, after enzymatic hydrolysis of the liquid fraction the xylose concentration was highest in the reactions with water impregnated samples compared to the acid impregnated samples (Figure 15). The opposite was observed with glucose; the acid impregnated straw had a higher initial concentration of glucose and this was also the case after enzymatic hydrolysis of the liquid fraction which correlates well with the content of C5 and C6 oligomers found in the liquids by MS analysis.

In addition to the MS analysis, the supernatants were also subjected to High Performance Liquid Chromatography to evaluate degradation components present in the supernatants. Furan and 5-hydroxyethyl furfarul was identified in all samples (data not shown). However, the absolute amount was not determined, but since both compounds appeared in all samples, the presence of these was not thought to contribute to the overall difference in hydrolysis of the substrates.

3.4.3 Enzymatic hydrolysis.

In general the glucose release was higher for hydrolysis reactions with barley straw than wheat straw (Figure 16, A and B, bars). The highest glucose concentration was obtained with acid impregnated, SE treated barley straw and the lowest was found for acid impregnated, SE treated wheat straw. For barley straw, the second highest glucose concentration was obtained with water impregnation and steam explosion of the substrate and the lowest was found for the hot water extracted substrate. However, based on yield...
Results and Discussion.

... in relation to the amount of potential glucose, the reverse was found; the highest yield was obtained with hot water extracted straw and the lowest yield was obtained with acid impregnated, steam exploded straw (Figure 16, filled diamonds). For wheat straw the highest yield and the highest glucose concentration was found for reactions with hot water extracted straw, but the results did not differ markedly. In the case of barley straw the harsher pretreatment resulted in slightly higher glucose concentration, but based on yields this pretreatment procedure was not as efficient as the least severe treatment (hot water extraction). In the case of wheat straw, the least severe pretreatment procedure proved most efficient both in terms of glucose release and yield. The differences in optimal pretreatment procedure might be related to differences in the straw structure and in fact the optimal pretreatment procedure did differ (Linde et al., 2006, Gracia-Aparicio et al., 2005); the SE conditions for barley straw was 220- 210°C, 5 min. and for wheat straw the SE was carried out at 190- 220 °C for 2.5 to 10 min. In comparison, the hot water extraction was a three-stage process with heating from 60 °C, 15 min. initially and a final heating step at 195 °C, 3 min. which was the same for both barley and wheat straw. These results suggest that acid impregnated, SE treated straw samples were efficiently pretreated and readily degradable for enzymatic hydrolysis resulting in a higher glucose concentration, however the higher yields obtained with water extracted samples suggest that this pretreatment might produce a substrate which contains less inhibitory compounds than the other samples wherefore this substrate is more efficiently hydrolysed and result in higher overall yields.

It was speculated that the low yield of the hydrolysis reactions could be related to enzyme deactivation during the 72 hours reaction and irreversible binding of the enzymes to lignin. Therefore we evaluated the effect of adding fresh enzyme after 24 hours reac-

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Figure 16. Glucose concentration (columns) and yield (black diamonds) (based on the potential glucose in the substrate as determined by acid hydrolysis) after 72 hours hydrolysis of pretreated substrates at 10 w/w % TS. Legends refer to acid impregnation followed by SE treatment of barley (Barley 1) and wheat straw (Wheat 1), water impregnation followed by SE treatment of barley (Barley 2) and wheat straw (Wheat 2), and hot water extraction of barley (Barley 3) and wheat straw (Wheat 3).
Results and Discussion.

It was shown that the samples with wheat straw benefited most from addition of extra enzyme compared to that of barley straw; the glucose release increased from 64 - 66 % in the wheat samples and 47 - 50 % in the barley samples and the water treated samples benefited more than the acid treated samples.

3.4.4 Conclusions.

The comparison between hydrolysis of barley and wheat straw showed that barley straw yielded a higher glucose concentration than wheat straw. In terms of pretreatment procedure it is suggested that the hot water extraction pretreatment is more efficient in producing a substrate that is most efficiently converted for both wheat and barley straw. The highest glucose concentration was obtained with acid impregnation and steam explosion but in terms of yield the hot water extraction procedure resulted in the highest yield. This might be related to the removal of hemicellulose from the lignocellulose during acid impregnation which might thus provide a more easily assessable cellulose moiety for enzymatic hydrolysis. However since this pretreatment procedure was expected to contain the highest amount of inhibitors the overall yield might lower compared to the other pretreatment procedures. The different pretreatment procedures also resulted in markedly different soluble oligomeric saccharide profiles which might be related to the use of acid during pretreatment. This supports the notion that addition of acid releases the majority of the hemicellulose as monosaccharides whereas water treatment releases the hemicellulose as oligomers since pentose oligomers were preferentially identified in the water treated samples.
4 Conclusions and outlook.

The results obtained in the study showed there is room for improvement of the enzymatic hydrolysis step in relation to hydrolysing steam exploded substrates for ethanol production.

In terms of improving the cellulase mixture it was shown that the well known cellulase product Celluclast and Novozyme 188 could be improved by addition of cellulase enzymes from other fungal sources which were able to boost the hydrolytic efficiency of Celluclast either by containing activities not present in Celluclast or by containing similar but more effective cellulase enzymes. It was shown that even addition of as little as 10 % enzyme protein from other fungal sources was able to significantly boost the activity of Celluclast at the optimal conditions for Celluclast at pH 5, 50 °C. The specific enzyme activities which were able to boost Celluclast was not identified but might consist of endoglucanase activities as evidenced by the higher amount of reducing ends produced when Avicel was used as substrate suggesting that when the fungal enzyme broths were added, more reducing ends from oligomers were created since the increase in reducing ends could not be accounted for by the amount of reducing ends arising from glucose and cellobiose. The boosting effect might also have been obtained by addition of hemicellulolytic activities not present in Celluclast. This is supported by more recent studies showing increasing hydrolytic efficacy of mixing Celluclast with multicomponent hemicellulase products (Berlin et al., 2005B; Berlin et al, 2006). These findings suggest that auxiliary enzymes, that is, activities other than celllylolytic enzymes, are important in relation to hydrolysis of lignocellulose. Since it has previously been shown that other filamentous fungi contain activities which are more effective in relation to lignocellulose degradation the strategy of identifying the activities that are more effective compared to the T. reesei enzyme mixture (Krogh et al., 2004; Berlin et al., 2004; Gusakov et al., 2006) seem highly feasible and could present a way to optimise the enzyme mixture best suited for lignocellulose hydrolysis.

In addition it was shown that the ratio of CBHI, CBHII, EGI and EGII representing the majority of the cellulase enzyme activities in Celluclast differed to some extent with respect to the pretreatment procedure in relation to whether the substrate was impregnated with acid or water prior to steam explosion or heating. In the case of water treated barley straw the most significant second component interaction effect arose from EGI and CBHI whereas for the acid impregnated barley straw the most pronounced effect arose from EGI and CBHII. It was shown that EGII activity was best omitted from the
Conclusions and outlook.

A mixture of monocomponent enzyme activities at a fixed enzyme protein dosage. It is concluded that the optimal distribution of the four monocomponent activities evaluated here are close to the distribution found in *T. reesei*. The absolute amount of the individual activities differed from that found in *T. reesei* i.e., EGI constitutes approximately 10% of the total enzyme from *T. reesei* but in the mixture design 27-38% was predicted to be optimal. These findings indicate that multicomponent enzyme mixtures are necessary for efficient lignocellulose hydrolysis even though only 5 activities are necessary for hydrolysis of pure cellulose. Future efforts to improve the multicomponent enzyme mixtures might be the way forward to create the better cellulase mixture for the biomass to ethanol process e.g. by mixing of a cellulase multicomponent with other hemicellulolytic multicomponent enzyme mixtures.

In an effort to increase the substrate loading in the hydrolysis reaction to a level relevant for a feasible lignocellulose to ethanol process the fed-batch strategy was evaluated. It was concluded that the fed-batch loading strategy was not particularly efficient in terms of reaching high glucose concentrations and yield when hydrolysing acid impregnated steam exploded barley straw in the conditions studied here. It was suggested that this was due to the high volume of liquid which was added to the fed-batch reactions in conjunction with more substrate. This diluted the glucose concentration markedly, coupled with the shorter reaction time the added substrate had in the hydrolysis reaction.

However, in terms of keeping the viscosity a low level to reduce the power input to efficiently mix the substrate and enzyme, the fed-batch strategy was efficient; the viscosity was kept at a low level throughout the reaction time and increased only transiently when more substrate was added. In contrast, the experiments which were loaded with all the substrate at the beginning of the reaction had a high initial viscosity which remained high until 24 hours reaction time. The effect of adding the entire enzyme loading based on final total dry matter in the fed-batch reactions resulted in high initial glucose yield and reduced the viscosity very rapidly compared to the reactions with all substrate loaded at the beginning of the reaction. However, the glucose yield did not reach comparable levels in the fed-batch reactions neither when the full enzyme loading was added in the beginning of the reaction nor when enzyme was added in conjunction with additional substrate. The fact that the glucose concentration decreased in the reaction loaded with the full enzyme loading in the beginning of the experiment was not related to more significant enzyme binding as the lignocellulose substrate was hydrolysed since the glucose concentration also decreased when fresh enzyme was added together with fresh substrate. Thus the fed-batch approach does not appear applicable in relation to the substrate loading scheme evaluated in the present study using acid impregnated, steam exploded bar-
Conclusions and outlook.

ley straw in terms of reaching high glucose concentration and yield, although the viscosity remained low during the reaction.

In relation to the pretreatment procedure and feedstock, it is concluded that barley acid impregnation followed by steam explosion results in higher glucose concentration but in terms of obtaining higher yield the hot water extraction procedure was more efficient for barley straw. In the case of wheat straw the hot water extraction procedure as also most efficient in terms of obtaining the highest yield whereas the three pretreatment procedures were equally efficient in terms of reaching the same glucose concentration during hydrolysis. Furthermore it was demonstrated that the acid impregnation prior to steam explosion resulted in markedly different soluble oligomeric saccharide profiles. This suggest that water impregnation prior to steam explosion releases the hemicellulose moiety as oligomers in contrast to acid treatment prior to steam explosion which results in almost complete solubilisation of the hemicellulose moiety (measured as the amount of xylose in the supernatant) with no soluble pentose oligomers.

Taking into account that no significant differences appeared between wheat and barley straw at different pretreatment conditions when evaluated by Celluclast and NS 188 hydrolysis it is suggested that the need to tailor the cellulolytic components might not be as important as optimising the spectrum of enzyme activities and include various hemicellulolytic enzyme activities. If so, then when applying a broad spectrum of enzyme activities might increase the overall glucose release and the overall differences relating to pretreated substrate structures might be more obvious and display major differences between feedstocks.

The recommendation for further improvements of the cellulase enzymes would thus be to continue investigating whether other fungal sources might contain cellulolytic activities to complement the high protein secreting T. reesei. Also, to search for fungal strains secreting cellulases displaying a higher catalytic effect or to improve/increase the activity of the T. reesei components by mutagenesis to increase the activity of specific components therein.

To further improve the enzymatic hydrolysis step, it might also be fruitful to further investigate exactly which accessory enzymes are necessary for the most efficient hydrolysis. These activities would most likely vary with respect to the pretreatment conditions when comparing e.g. hot water extracted lignocellulose where the hemicellulose is released as oligomers which will require and even broader spectrum of hemicellulosic activities than more severely pretreated substrates where the hemicellulose constituents are released as monosaccharides. The pretreatment conditions are in turn dependent on what the objective of the whole process is; if only the cellulose moiety is concerned then the more
Conclusions and outlook.

severely pretreated material result in the higher glucose release. However, if the hemicellulose is intended to be used as well, then a low severity pretreatment would be recommended since this would mean less degradation of the hemicellulose constituents.

In view of the overall process leading to ethanol production it is obvious that the substrate loading must be relatively high. The fed batch strategy might prove beneficial if optimised both with respect to substrate additions and also substrate addition in conjunction with additional enzyme addition. Since it has been shown that EG activity decreases viscosity it could be envisaged that addition of more EG relative to CBH activity might also prove beneficial since the EG would generate more active sites for the CBH activity.

This would however require multiple enzyme addition steps but might prove to be an interesting alternative to addition of the whole multicomponent enzyme mixture.

The low dry matter content of the pretreated substrates evaluated in the Ph. D. project presented a problem since this resulted in a low final glucose concentration. Part of the solution might relate to the specific pretreatment step. If it would be possible to increase the substrate loading more often and in lower increments of substrate then it might be possible to overcome the dilution effect observed in the present PhD project. Since results have previously indicated that simultaneous saccharification fermentation is more efficient in relation to the ethanol yield than the separate hydrolysis and fermentation step, a hybrid scenario might be envisaged; with a pre saccharification step at relatively low dry matter loading to produce a high glucose concentration fast, then add yeast to initiate the fermentation and then continue to increase the substrate loading to relieve end product inhibition and continue hydrolysis. Compared to results by Varga et al. 2004 where it was also observed that the hydrolysis reaction decrease in fed-batch reaction at higher substrate loading, this was also attributed to insufficient mixing but also increasing amount of inhibitory compounds in the pretreated substrates. In the hot water extracted straw, the acid and also the water impregnated steam exploded straw evaluated here, both furan and 5-hydroxykethylefurfarul was identified. Since inhibitory compounds present in pretreated lignocellulose inhibit both fermentation and saccharification, it is suggested that a fed batch strategy might be more effective if washed substrate was utilised. In relation to an industrial biomass to bioethanol it is however imperative to operate at high substrate loading and the washing approach might not be fruitfull.

The main conclusions in relation to improving the enzymatic hydrolysis step in the biomass to bioethanol process are summarised as:
Conclusions and outlook.

The enzyme mixture for the most efficient hydrolysis of steam exploded straw can be optimised and improved, possibly by addition of hemicellulolytic enzyme activities or by optimising the cellulolytic enzyme mixture.

A fed-batch approach to increase the substrate loading and keep the viscosity of the reactions low during the enzymatic hydrolysis was effective in relation to maintaining a low level of viscosity through out the reaction.

In order to reach high glucose yields in fed-batch reactions the timing of addition of extra substrate or application of substrate with higher dry matter needs to be refined.

Acid and water treatment differ mainly with respect to the content of pentose oligomers in the liquid fraction after pretreatment. Acid treatment prior to steam explosion results in almost complete release of the hemicellulose as monosaccharides whereas the water impregnation results in oligomers of the hemicellulose.
Conclusions and outlook.
5 References.


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6 Publications

Paper 1
Evaluation of minimal *Trichoderma reesei* cellulase mixtures on differently pretreated barley straw substrates.
*Biotechnology progress, 23, 1270- 1276.*

Paper 2
Comparison of different pretreatment strategies for degradation of wheat and barley straw
*Applied Biochemistry and Biotechnology, 143, 284-296.*

Paper 3
Effects of substrate loading on enzymatic hydrolysis and viscosity of pretreated barley straw.
*Applied Biochemistry and Biotechnology, 143, 27-40.*

Paper 4
L. Rosgaard, S. Pedersen, J. R. Cherry, P. Harris, A. S. Meyer (2006).
Efficiency of new fungal cellulase systems in boosting enzymatic degradation of barley straw lignocellulose.
*Biotechnology progress, 22, 493- 498.*
Evaluation of Minimal Trichoderma reesei Cellulase Mixtures on Differently Pretreated Barley Straw Substrates

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The commercial cellulase product Celluclast 1.5, derived from Trichoderma reesei (Novozymes A/S, Bagsværd, Denmark), is widely employed for hydrolysis of lignocellulosic biomass feedstocks. This enzyme preparation contains a broad spectrum of cellulolytic enzyme activities, most notably cellobiohydrolases (CBHs) and endo-1,4-β-glucanases (EGs). Since the original T. reesei strain was isolated from decaying canvas, the T. reesei CBH and EG activities might be present in suboptimal ratios for hydrolysis of pretreated lignocellulosic substrates. We employed statistically designed combinations of the four main activities of Celluclast 1.5, CBHI, CBHII, EGI, and EGII, to identify the optimal glucose-releasing combination of these four enzymes to degrade barley straw substrates subjected to three different pretreatments. The data signified that EGI activity is not required for efficient lignocellulose hydrolysis when efficient of this activity occurs at the expense of the remaining three activities. The optimal ratios of the remaining three enzymes were similar for the two pretreated barley samples that had been subjected to different hot water pretreatments, but the relative levels of EGI and CBHII activities required in the enzyme mixture for optimal hydrolysis of the acid-impregnated, steam-exploded barley straw substrate were somewhat different from those required for the other two substrates. The optimal ratios of the cellulolytic activities in all cases differed from that of the cellulases secreted by T. reesei. Hence, the data indicate the feasibility of designing minimal enzyme mixtures for pretreated lignocellulosic biomass by careful combination of monocomponent enzymes. This strategy can promote both a more efficient enzymatic hydrolysis of (ligno)cellulose and a more rational utilization of enzymes.

Introduction

The application of cellulases for conversion of lignocellulosic biomass to ethanol has been investigated for a long time, and it is now well-known that a mixture of different cellulolytic activities is necessary for the hydrolysis of the cellulose moiety into glucose for fermentation into ethanol (1−3). For degradation of pure cellulose, the requirement for the concerted action of cellobiohydrolase (CBH) (EC 3.2.1.91), endo-1,4-β-glucanase (EG) (EC 3.2.1.4), and β-glucosidase (BG) (EC 3.2.1.21) activities has been amply documented (3−5). Trichoderma reesei, now one of the most well-known and most studied cellulase-producing fungi, was originally isolated from decaying canvas during World War II (6). The organism secretes five different endoglucanases, EG I−V, or Cel7B, Cel5A, Cel12A, Cel61A, and Cel45A, respectively, (the latter categorization corresponding to the nomenclature of glycosyl hydrolase families) and two types of CBH activities, CBHI (Cel7A) and CBHII (Cel6A), as well as a number of xylanases and at least one β-xylanase enzyme (Table 1). (17). The reason for T. reesei to produce isomers of the cellulases is not clear yet; however, it is known that the two CBH activities, CBHII and CBHH, have slightly different topology and catalyze the hydrolysis of the cellulose processively from the reducing end and the non-reducing end of the cellulose, respectively, through a tunnel-shaped active site (18, 19).

As evaluated from the relative amount of protein, the CBHII is the main enzyme produced by T. reesei, followed by CBHI (Table 1). Similarly, EGI and EGII are the two major EG

### Table 1. Approximate Relative Protein Levels of Cellulolytic and Xylanolytic Enzymes Produced by Trichoderma reesei (Rut C-30)*

<table>
<thead>
<tr>
<th>enzyme</th>
<th>% of total protein</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cel7A (CBHI)</td>
<td>40−60</td>
<td>(7, 8)</td>
</tr>
<tr>
<td>Cel6A (CBHII)</td>
<td>12−20</td>
<td>(7, 9)</td>
</tr>
<tr>
<td>Cel7B (EGI)</td>
<td>5−10</td>
<td>(8, 10)</td>
</tr>
<tr>
<td>Cel5A (EGII)</td>
<td>1−10</td>
<td>(8, 11)</td>
</tr>
<tr>
<td>Cel12A (EGIII)</td>
<td>&lt;1−5</td>
<td>(8, 11)</td>
</tr>
<tr>
<td>Cel61A (EGIV)</td>
<td>&lt;1</td>
<td>(12)</td>
</tr>
<tr>
<td>Cel45A (EGV)</td>
<td>&lt;5</td>
<td>(8, 13)</td>
</tr>
<tr>
<td>BG1</td>
<td>1−2</td>
<td>(8, 13)</td>
</tr>
<tr>
<td>BG2</td>
<td>&lt;1</td>
<td>(13)</td>
</tr>
<tr>
<td>Xylanase I (GH 11)</td>
<td>n.d.</td>
<td>(14)</td>
</tr>
<tr>
<td>Xylanase II (GH 11)</td>
<td>n.d.</td>
<td>(14)</td>
</tr>
<tr>
<td>Xylanase III (GH 10)</td>
<td>n.d.</td>
<td>(15)</td>
</tr>
<tr>
<td>Xylanase IV (GH 5)</td>
<td>n.d.</td>
<td>(15)</td>
</tr>
<tr>
<td>β-Xylanosidase (GH 3)</td>
<td>n.d.</td>
<td>(16)</td>
</tr>
</tbody>
</table>

* Relative protein levels vary somewhat depending on growth medium and cultivation conditions. For cellulolytic enzyme activity abbreviations, please see text. ** GH: glycoside hydrolase family. * n.d.: not determined.

*This enzyme has been characterized from T. reesei PC-3-7 and has high homology with GH 10 enzymes (15).
substrate adsorption phenomena of selected *T. reesei* secreted by cellulase enzyme activities necessary for lignocellulose hydrolysis. The optimal mutual ratio of such a minimal mixture of barley straw substrates in a rational design template to identify the specific activity of CBHII is approximately twice that of CBHII when measured on Avicel and end, respectively, the specific activity of EGI. Hence, CG activity has to be applied exogenously to supplement the Celluclast 1.5L and to overcome product inhibition from cellobiose, and this is often accomplished by addition of Novozym 188, a BG preparation from *Aspergillus niger*.

The synergy of reconstituted cellulase mixtures based on combining purified EG and CBH activities from *T. reesei* and other fungi has been described previously. Some of these enzyme mixtures have been shown to perform as well or even surpass the performance of crude *T. reesei* cellulase enzyme mixtures in converting various pure cellulose substrates to glucose. A few studies using minimal mixtures of purified cellulases have been performed on lignocellulosic substrates. These studies have aimed at either assessing the performance of novel fungal cellulases from other cellulosytic fungi or at, for example, examining enzyme-substrate adsorption phenomena of selected *T. reesei* enzymes.

In this study we compared the effect of the addition of the four main activities from *T. reesei* on differently pretreated barley straw substrates in a rational design template to identify the optimal mutual ratio of such a minimal mixture of *T. reesei* cellulase enzyme activities necessary for lignocellulose hydrolysis. The *T. reesei* enzymes CBHI, CBHII, EGI, and EGII were chosen on the basis of the observations that they together constitute the major portion of the enzymes (based on protein) secreted by *T. reesei*. In all experiments BG activity was supplemented in order to avoid any product inhibition from cellobiose.

**Materials and Methods**

Substrates. Barley straw was grown and harvested in 2006 in Funen, Denmark. The straw was kindly pretreated by DONG Energy (Danish Oil and Natural Gas Energy), Denmark and by the Department of Chemical Engineering, Lund University, Sweden, respectively. At Lund University, steam explosion was done on barley straw that had been chopped to pieces of 2 cm in length and (Barley 1) sprayed with 1% H$_2$SO$_4$, incubated for 1 h, and subjected to steam explosion at 220 °C for 5 min (optimal conditions according to ref 30) or (Barley 2) soaked in water at 70% dry matter for 1 h followed by steam explosion at 210 °C for 5 min (optimal conditions for steam explosion of barley straw according to ref 31). In the DONG pretreatment (Barley 3), whole bales of barley straw were subjected to hot water extraction in three stages. First the straw was loaded at 16% w/w dry matter and held at 60 °C for 15 min, after which the liquor was removed. This was followed by treatment at 180 °C for 10 min and finally by 3 min at 195 °C. Since the hydrolysis experiments were carried out on 2 g scale, the pretreated barley straw arising from the DONG pretreatment was dried at room temperature for 7 days and subsequently milled and sieved into a length of 2 mm.

**Enzymes.** The enzyme system to be replaced consisted of Celluclast 1.5L derived from *T. reesei* (in the following referred to as Celluclast) supplemented with Novozym 188 (NS 188) derived from *A. niger* (Novozymes A/S, Bagsværd, Denmark). The filter paper activity of the Cellulast was 68 FPU g$^{-1}$ (FPU = filter paper units) according to the standard assay procedure provided by the U.S. National Renewable Energy Laboratory (32). The activity of Novozym 188 (NS 188), derived from *A. niger*, was 246 CBU g$^{-1}$ (CBU = cellobiose units). The CBU activity was determined by measuring glucose production on cellobiose at 40 °C, pH 5 (provided by Novozymes A/S).

**Purification of *T. reesei* Cel7A (CBHI).** Cellulase was buffer-exchanged into 20 mM Tris/HCl, pH 8.0 using a Pall Filtron concentrator with an Omega 10 KD cutoff membrane. The resulting material was then purified over a Q-Sepharose Fast-Flow column (GE Healthcare) in the same buffer over a linear 0–500 mM sodium chloride gradient. Fractions were pooled on the basis of 3DS–PAGE and then desalted into 20 mM Tris/HCl, pH 8.0 as before. The resulting material was then purified over a Mono Q column (GE Healthcare) in the same buffer over a linear 0–500 mM sodium chloride gradient. Fractions were pooled on the basis of 3DS–PAGE to give the desired *T. reesei* CBHI as a single band (~95% pure) at ~60 kD.

**Production of Cel7B, Cel5A, and Cel6A (EGI, EGII, and CBHII).** The *T. reesei* EGI and EGII proteins were expressed separately in a recombinant *Aspergillus oryzae* host and CBHII was expressed in a recombinant *Fusarium venenatum* strain. Fermentations of these were done in 3-L glass-jacketed bioreactors (Applikon Biotechnology, Foster City, CA) equipped with Rushton impellers on defined media containing yeast extract at 10 g L$^{-1}$ (*A. oryzae* strain), soy concentrate (20 g L$^{-1}$) (**F. venenatum**), sucrose (20 g L$^{-1}$) (**F. venenatum**), (NH$_4$)$_2$SO$_4$ (5 g L$^{-1}$), KH$_2$PO$_4$ (2 g L$^{-1}$), CaCl$_2$·2H$_2$O (0.5 g L$^{-1}$), MgSO$_4$·7H$_2$O (2 g L$^{-1}$), citric acid (1 g L$^{-1}$), K$_2$SO$_4$ (2 g L$^{-1}$), antifoam (0.5 mL L$^{-1}$), and a trace metals solution (0.5 mL L$^{-1}$) composed of FeSO$_4$·7H$_2$O (13.8 g L$^{-1}$), ZnSO$_4$·7H$_2$O (14.3 g L$^{-1}$), MnSO$_4$·H$_2$O (8.5 g L$^{-1}$), CuSO$_4$·5H$_2$O (2.5 g L$^{-1}$), and citric acid (3 g L$^{-1}$). The total fermentation period was 185 h with air added at a rate of 1vvm, impellers rotating at 1100–1300 rpm, and maltose fed at 0–0.44 g L$^{-1}$ h$^{-1}$, at 29 °C, pH 6.25 (**F. venenatum**) and at 34 °C, pH 6.1 (*A. oryzae* strain). The pH was controlled using an Applikon 1030 control system (Applikon Biotechnology, Foster City, CA) to a set point of the selected pH of ± 0.25 (**F. venenatum**) and ± 0.1 (*A. oryzae*).

In each case the whole broth was harvested after 8 days of fermentation and centrifuged at 3000 × g to remove biomass; the resulting supernatant was sterile filtered and delivered as an enzyme sample.

**Protein.** Protein contents of the enzyme samples were determined by the Micro BCA kit from Pierce Biotechnology Inc. (Rockford, IL).

**Enzyme Hydrolysis Experiments.** For the enzymatic hydrolysis experiments substrate slurries were prepared by weigh-
ing off substrate and buffer. Slurries were subsequently left to rehydrate at 4 °C for 24 h before use. All hydrolysis reactions were carried out at 1% (w/w) dry matter at pH 5, 50 °C. A benchmark reaction was carried out using a protein loading of 6.5 FPU·g dry matter⁻¹ of Celluclast equivalent to 11 mg of the Celluclast enzyme g dry matter⁻¹ and supplemented with 5 mg NS 188 enzyme protein g dry matter⁻¹. The protein levels employed in the substitution experiments with purified monocomponent cellobiohydrolase I, II (EC 3.2.1.91) and endoglucanase I and II (EC 3.2.1.4) were accomplished to match the protein loading of 11 mg Celluclast g dry matter⁻¹ (equal to 6.5 FPU·g dry matter⁻¹), that is, the sum of protein from the four monocomponents per designed reaction, added according to the mixture design template described below, was equal to 11 mg·g dry matter⁻¹, and this dosage was then supplemented with 5 mg NS 188 protein·g dry matter⁻¹.

The Celluclast substitution experiments were carried out in a mixture design template created and evaluated by use of SAS JMP 5.1 (The SAS Institute, 2003). The template consisted of 56 different reactions comprising designed dosage combinations of each of the four cellulolytic enzymes: each enzyme dosage was varied from 0 to 100% in increments of 20% (i.e., 0%, 20%, 40%, 60%, 80%, and 100%) of the total enzyme loading for each of the four cellulolytic enzymes: each enzyme dosage was varied from 0 to 100% in increments of 20% (i.e., 0%, 20%, 40%, 60%, 80%, and 100%) of the total enzyme loading. The template was run twice for each substrate.

**Analysis of Substrate Carbohydrate Compositions and Glucose and Xylose Release.** The structural carbohydrates compositions in the differently pretreated barley straw substrates were determined after acid hydrolysis according to the U.S. National Renewable Energy Laboratory (NREL) standard method (55). Glucose, xylose, arabinose, and galactose were separated by high performance anion exchange chromatography (HPAEC) on a Dionex BioLC system equipped with a CarboPac PA1 column (4 × 250 mm) (Dionex Denmark A/S, Hvidovre, DK) and a CarboPac PA1 guard column (4 × 50 mm). Samples were eluted isocratically with 0.01 M KOH at a flow rate of 1 mL per minute, and analytes were detected and quantified against standard curves of the relevant monosaccharides by electrochemical detection in a pulsed amperometric detection mode as described previously (34).

**Results and Discussion**

**Composition of Differently Pretreated Barley Straw Biomass Stocks.** On the basis of the measured levels of arabinose and xylose (only negligible amounts of galactose were detected) (Table 2), the hemicellulose was estimated to make up approximately 5–12% of the dry matter, varying somewhat between the different pretreatments, and as expected to be mostly contributed by xylan (Table 2). The glucose potential, defined as the level of glucose obtained by the acid hydrolysis of the pretreated substrates, was relatively similar for both of the steam-exploded substrates and ranged from 57% to 61% of the DM, i.e., 570–610 g glucose·kg⁻¹ substrate dry matter, whereas the hot water extraction pretreatment resulted in a lower glucose potential of 41% of the DM (Table 2).

**Benchmark Enzymatic Hydrolysis.** The glucose release as a function of time of the acid-treated and water-treated barley straw subjected to steam explosion using benchmark conditions with Celluclast and NS 188 is shown in Figure 1. During the enzymatic hydrolysis the glucose release occurred more rapidly in the reactions with the acid-impregnated, steam-exploded straw than in the water-impregnated, steam-exploded and in the hot-water-extracted straw (Figure 1A). After 24 h of enzymatic reaction, the glucose release was higher for the acid-treated straw than for the water-treated straw and the hot-water-extracted straw. However, after an additional 24 h of enzymatic hydrolysis, i.e., after a total enzymatic reaction of 48 h, the glucose releases were similar in both steam-exploded substrates (Figure 1A). Compared to the glucose potential in each of the pretreated substrates (Table 2), the final glucose release after 48 h reaction was equivalent to 81% and 78% cellulose conversion, calculated as (g glucose 48 h·g potential glucose⁻¹ × 100), for the two steam-exploded substrates, i.e., acid- and water-impregnated, respectively, but corresponded to 90% conversion for the hot-water-extracted straw. Since the marginal increase in the glucose release from 24 to 48 h was low, the subsequent experiments in which the benchmark enzyme loading was substituted with purified cellulase components, were run for 24 h.

In accordance with the application of acid during pretreatment solubilizing a large part of the hemicellulose to monosaccharides, the xylose releases were higher in the reactions with water-impregnated, steam-exploded straw and hot-water-extracted straw than in those with acid-pretreated straw (Figure 1B). The maximal yields of xylose, (g xylose 48 h·g potential xylose⁻¹ × 100, from the potential xylose levels (Table 2) obtained varied from 65% to 700% of the “theoretical” maximum assessed by acid hydrolysis (Table 2) and was highest for the water-impregnated, steam-exploded straw. The yield obtained above 100% of the “theoretical maximum” is presumably a result of the “theoretical maximum” in fact being too low and underestimated, which we presume is related to degradation of xylose during the acid hydrolysis applied for determination of the structural carbohydrates in the substrate.

**Monocomponent Enzyme Mixtures.** The EGI, EGII, CBHI, and CBHII were applied as pure enzymes in mutual ratios ranging from 0 to 100% following a mixture design template incorporating all four activities. Therefore, no bias was taken to the fact that the main activity in the original Celluclast cellulase mixture is CBHI based on the protein level (Table 1). The experiments using monocomponent enzymes EGI, EGII, CBHI, and CBHII showed that only EGI and CBHII significantly affected glucose release positively as single factors and only on the steam-exploded substrates, whereas both EGI and CBHII contributed non-significantly as single factors to the glucose release (Table 3). In contrast, the two-factor interaction effects ranged from significant to highly significant for all the possible two-factor combinations on all substrates; the only exception was the EGII × CBHII effect on the hot-water-extracted wheat substrate (Table 3). When comparing the responses on the differently pretreated substrates, the hot-water-extracted substrate gave the lowest number of main and interactive response effects to the enzymatic treatments (Table 3).

These results are in complete agreement with previous work showing that the cellulolytic degradation of cellulose and lignocellulose requires the concerted action of different cellulases (5, 4, 28). The synergistic effects of cellobiohydrolases with endoglucanases are particularly well-documented on both lignocellulosic substrates and on pure cellulose substrates (28, 35). The extent of synergism between cellobiohydrolases and endoglucanases is related to the nature of the substrate and the
extent of substrate degradation, i.e., cellobiohydrolases have a more pronounced effect than endoglucanases on relatively crystalline substrates, whereas endoglucanases exert most effect on less crystalline substrates (3).

As evaluated from the values of the multiple linear regression coefficients, the extent of the different interactions varied among the differently pretreated substrates. Hence, for the acid-impregnated and steam-exploded straw the most pronounced interactions appeared to be between EGI and CBHII (coefficient 24.8, \(p < 0.0001\), Table 3) and between CBHII and CBHII (coefficient 24.2, \(p < 0.0001\), Table 3). However, in the case of the water-treated straw substrates, the most pronounced interactions, i.e., the highest and most significant coefficients, were found for the EGI \(\times\) CBHII interaction (Table 3, bold). Together, CBHII and EGI usually make up the larger portion of the enzymes secreted by *T. reesei* (cf. Table 1), suggesting that indeed the ratio in which these enzymes are produced is positively related to the effect they have on lignocellulose degradation. For the acid-impregnated, steam-exploded barley straw the EGI \(\times\) CBHII interaction was also highly significant, but on this substrate the positive interaction coefficient was higher for the EGI \(\times\) CBHI interaction, indicating that on this acid substrate the latter interaction had a more pronounced influence on the glucose yields than the EGI \(\times\) CBHII interaction (Table 3). The multivariate analyses of the data also verified a significantly positive interaction on the glucose release between the two cellobiohydrolases, CBHI \(\times\) CBHII (Table 3), indicating that on pretreated substrates the co-action of both of the cellobiohydrolases is advantageous.

**Optimal Mixture.** On the acid-impregnated and steam-exploded straw, the optimal mixture of monocomponent enzymes (+NS 188) resulted in a glucose release of 3.7–3.8 g·L\(^{-1}\).

![Figure 1.](image)

(A) Glucose release (g·L\(^{-1}\)) and (B) xylose release (g·L\(^{-1}\)) during hydrolysis of acid-impregnated, steam-exploded barley straw (Ω); water-impregnated, steam-exploded barley straw (●); and hot-water-extracted straw (×) using the benchmark enzyme loading of Celluclast and NS 188 (11 mg Celluclast enzyme protein·g dry matter\(^{-1}\) and 5 mg NS 188 enzyme protein·g dry matter\(^{-1}\)).

**Table 3. Main and Interaction Effects of Monocomponent Enzymes in the Hydrolysis Reactions at Maximal Glucose Release**

<table>
<thead>
<tr>
<th>effects</th>
<th>acid impregnation, SE (r^2 = 0.8, \text{RMSE} = 0.5)</th>
<th>water impregnation, SE (r^2 = 0.8, \text{RMSE} = 0.2)</th>
<th>hot water extraction (r^2 = 0.9, \text{RMSE} = 0.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGI</td>
<td>1.0^{0.7}</td>
<td>1.0^{0.7}</td>
<td>0.7^{0.7}</td>
</tr>
<tr>
<td>EGII</td>
<td>1.1^{0.7}</td>
<td>1.6^{0.8}</td>
<td>1.9^{0.7}</td>
</tr>
<tr>
<td>CBHII</td>
<td>0.8^{0.7}</td>
<td>1.0^{0.8}</td>
<td>0^{0.7}</td>
</tr>
<tr>
<td>CBHI</td>
<td>3.1^{0.8}</td>
<td>3.0^{0.8}</td>
<td>0^{0.7}</td>
</tr>
<tr>
<td>EGI (\times) EGII</td>
<td>0^{0.7}</td>
<td>7.1^{3.0}</td>
<td>0^{0.7}</td>
</tr>
<tr>
<td>EGI (\times) CBHII</td>
<td>24.8^{2.9}</td>
<td>19.5^{2.2}</td>
<td>13.6^{6.0}</td>
</tr>
<tr>
<td>EGII (\times) CBHII</td>
<td>17.8^{3.0}</td>
<td>10.5^{3.2}</td>
<td>0^{0.7}</td>
</tr>
<tr>
<td>EGI (\times) CBHI</td>
<td>14.0^{3.0}</td>
<td>27.8^{4.2}</td>
<td>31.1^{5.9}</td>
</tr>
<tr>
<td>CBHI (\times) CBHII</td>
<td>13.0^{3.0}</td>
<td>10.1^{2.9}</td>
<td>25.8^{5.3}</td>
</tr>
<tr>
<td>CBHI (\times) CBHII</td>
<td>24.2^{3.0}</td>
<td>19.0^{3.0}</td>
<td>25.5^{4.4}</td>
</tr>
</tbody>
</table>

* Probability of effects \((F-test): F < 0.0001 = \(*\*\), 0.0001 < F < 0.001 = \(*\), 0.01 < F < 0.05 = \(*\), F > 0.05 = \(\text{NS}\) (non-significant). The data are expressed as the percentage each monocomponent enzyme provides of the total, maximal glucose concentration in the hydrolysis ± the standard deviation calculated from the parameter estimates provided in the model for each substrate type. The model fit \((R^2)\) and the standard deviation of the residual error \((\text{RMSE})\) is shown.
The glucose release obtained with the mono-component mixture was equivalent to 56% conversion of the potential glucose present in the acid-treated, steam-exploded straw (Table 2). Apparently, within the experimental setup and enzyme protein dose evaluated here, EGII was better left out of the enzyme mixture since addition of EGII at the expense of adding less of the remaining components resulted in lower glucose release. The predicted optimal enzyme mixture in percent of the total protein dose EGI:CBHI:CBHII was then 0.27:0.27:0.47, equivalent to a relative percentage distribution of activities in the mixture of ∼25%:25%:50% (Figure 2). Comparedly, for water-impregnated steam-exploded barley straw, the conclusion was similar with regard to the composition of the optimal enzyme mixture being EGI:CBHI:CBHII. The optimal distribution in percent of the total protein dose being 0.38:0.17:0.45 (Figure 2, “Water + SE”). This optimal mixture was predicted to yield a glucose concentration of 2 g L⁻¹ corresponding to 33% conversion of the glucose present in the substrate.

The optimal enzyme composition for degradation of the hot-water-extracted barley straw was 0.37:0.20:0.43 (Figure 2, “Hot water”). Interestingly, even though the pretreatments were inherently different, the optimal mixtures to convert the cellulose in the substrate residues were almost similar for the differently pretreated substrates and virtually identical for the two water-pretreated substrates. This conclusion suggests that the nature of the cellulose substrates resulting after the different pretreatments were in essence quite alike.

The results regarding the optimal combination of monocomponent activities were significantly different from those previously published for the biodegradation of crystalline bacterial cellulose ribbons by optimized mixtures of recombinant cellulases from *Humicola insolens* (27). Obviously, both the substrate and the fungal source of the enzymes differed, which might be the main reason for this difference. The data obtained thus indicate that cellulase compositions optimized on pure, crystalline cellulose may not be optimal for pretreated, lignocellulosic substrates.

In a previous study with *Chrysosporium lucknowense* cellulases it was shown that the glucose release obtained after treatment of pure cellulose with an artificial mixture of cellulases EGI, EGII, CBHI, and CBHII from *C. lucknowense* surpassed

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**Figure 2.** Prediction profiles for the glucose release from acid-soaked, steam-exploded straw (Acid + SE), water-soaked, steam-exploded straw (Water + SE), and hot-water-extracted straw (Hot water) as a response to different mixtures of CBHI, CBHII, EGI, and EGII. The predicted values of the optimal ratio of each monocomponent are shown in red. The reaction conditions were pH 5, 50 °C using a total protein loading of 11 mg monocomponent activity g dry matter⁻¹ supplemented with 5 mg NS 188 protein g dry matter⁻¹.
the hydrolytic efficacy of crude cellulase preparations from \textit{T. reesei} (26). In the present work the highest cellulose conversion percentage obtained after 24 h of enzymatic reaction using the optimal mixture of the monocomponent \textit{T. reesei} enzymes, as evaluated from glucose release on the acid-impregnated, steam-exploded barley straw, corresponded to \textasciitilde 79\% of the conversion obtained with the benchmark treatment of Celluclast and NS 188, i.e., with NS 188 added at 5 mg g\textsuperscript{-1} dry matter of the substrate and Celluclast at 11 mg protein g\textsuperscript{-1} dry matter of the substrate (Figure 3). In case non-cellulase accessory enzymes are required for optimal hydrolysis of lignocellulose (16), this requirement may explain why the yields obtained with the different pretreated substrates differed from those obtained with the full Celluclast treatment. Hence, the acid-impregnated, steam-exploded substrate, which had a lower amount of residual hemicellulose than the water-extracted straw substrates (Table 2), gave a relatively better yield with the monocomponent enzyme mixture (79\% of that of Celluclast) compared to the water-extracted straw sample (50\% of the Celluclast yield for the water-impregnated, steam-exploded straw); with the hot-water-extracted straw, the yields obtained compared to 80\% of those obtained with the full Celluclast (data not shown).

\textbf{Evaluation of the Effect of \( \beta \)-Glucosidase Activity.} There was hardly any difference in the composition of the optimal enzyme mixture in relation to hydrolysis of the three different substrates (Figure 2). Further optimization of the \( \beta \)-glucosidase loading in the form of NS 188 was carried out on the acid-treated, steam-exploded barley straw substrate. The NS 188 loading was varied from 0 to 5\% by weight of the dry matter, i.e., 0\%–50 mg enzymatic protein g\textsuperscript{-1} dry matter, loaded in the reaction. The data indicated that the glucose release with the optimal monocomponent mixture could be increased by \textasciitilde 26\% when the NS 188 dosing was increased by 5- to 10-fold (Figure 3). However, the results also signified that the yields obtained with the equivalent, full Celluclast + NS 188 addition could not be surpassed by increasing the NS 188 addition to the monocomponent cellulase mixture. In turn, this implied that cellulobiase accumulation was not significantly higher with the monocomponent mixture than with Celluclast, despite the relatively higher proportion of cellobiohydrolases in the optimized monocomponent mixture.

\textbf{Conclusions}

Even though the level of glucose release obtained with treatment of a blend of only the four main cellulolytic activities secreted by \textit{T. reesei} did not reach the level obtained with the comparable full Celluclast treatment, the results confirmed our initial proposition that an altered proportional composition of the main \textit{T. reesei} cellulases (present in the enzyme preparation Celluclast 1.5 L) was more optimal for enzymatic hydrolysis of cellulose in pretreated barley straw biomass than the composition secreted by \textit{T. reesei}. We believe that the approach involving selection and combination of only the enzyme activities having maximal impact on the enzymatic degradation of the lignocellulosic substrate in question has the potential to promote both a more efficient substrate degradation and a more rational enzyme utilization, providing for an improved economy in the conversion of lignocellulosic biomass feedstocks to biofuels.

\textbf{Acknowledgment}

This research was partially financed by the European Commission Framework V, contract no. NER/2001/685 (The Balsafuent Bioethanol Project), Danish Oil and Natural Gas Energy, Guido Zacchi and Marie Linde, Lund University, Sweden, are thanked for their assistance in pretreating the barley and wheat straw.

\textbf{References and Notes}


Received September 20, 2007. Accepted October 22, 2007.
Comparison of Different Pretreatment Strategies for Enzymatic Hydrolysis of Wheat and Barley Straw

Lisa Rosgaard · Sven Pedersen · Anne S. Meyer

Published online: 18 August 2007
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Abstract In biomass-to-ethanol processes a physico-chemical pretreatment of the lignocellulosic biomass is a critical requirement for enhancing the accessibility of the cellulose substrate to enzymatic attack. This report evaluates the efficacy on barley and wheat straw of three different pretreatment procedures: acid or water impregnation followed by steam explosion versus hot water extraction. The pretreatments were compared after enzyme treatment using a cellulase enzyme system, Celluclast 1.5 L® from Trichoderma reesei, and a β-glucosidase, Novozyme 188 from Aspergillus niger. Barley straw generally produced higher glucose concentrations after enzymatic hydrolysis than wheat straw. Acid or water impregnation followed by steam explosion of barley straw was the best pretreatment in terms of resulting glucose concentration in the liquid hydrolysate after enzymatic hydrolysis. When the glucose concentrations obtained after enzymatic hydrolyses were related to the potential glucose present in the pretreated residues, the highest yield, ~48% (g g⁻¹), was obtained with hot water extraction pretreatment of barley straw; this pretreatment also produced highest yields for wheat straw, producing a glucose yield of ~39% (g g⁻¹). Addition of extra enzyme (Celluclast 1.5 L®+Novozyme 188) during enzymatic hydrolysis resulted in the highest total glucose concentrations from barley straw, 32–39 g L⁻¹, but the relative increases in glucose yields were higher on wheat straw than on barley straw. Maldi-TOF MS analyses of supernatants of pretreated barley and wheat straw samples subjected to acid and water impregnation, respectively, and steam explosion, revealed that the water impregnated + steam-explored samples gave a wider range of pentose oligomers than the corresponding acid-impregnated samples.

Keywords Lignocellulose · Enzymatic hydrolysis · Glucose yield · Pretreatment
Introduction

Production of bioethanol from lignocellulosic biomass feedstocks has attracted much attention as an alternative route to expand the current starch- and sucrose-based production of ethanol for the manufacture of transport fuels to replace fossil fuels.

One of the first requirements in the utilization of lignocellulose for production of ethanol is to efficiently produce a fermentable hydrolysate rich in glucose from the cellulose present in the feedstock. Employment of enzymes for the hydrolysis of the lignocellulose is considered the prospectively most viable strategy to provide a cost-efficient, environmentally friendly process, and to avoid generation of byproducts that may inhibit the subsequent fermentation [1–3]. However, the physico-chemical and structural composition of native lignocellulose hinders direct enzymatic hydrolysis of the cellulose and hemicellulose present in lignocellulosic biomass. To increase the accessibility of the cellulose to enzymatic attack the lignocellulosic substrates therefore have to undergo a physico-chemical pretreatment before the enzymatic hydrolysis step [4].

Several different pretreatment processes are efficient in providing a relatively easily degradable substrate [4–6]. In brief, pretreatment may involve a mechanical step for reducing the substrate particle size of the native straw followed by one or more steps of heating and wetting the straw in the presence of a catalyst [4]. Acid, alkali or water itself at high temperature can be used as catalysts. With acid pretreatment, the hemicellulose present in the straw is solubilized, in effect producing a solid fraction consisting of mainly cellulose and lignin. The wet-oxidation and alkaline-based methods are generally relatively more effective at solubilizing lignin, but leaves behind much of the hemicellulose in an insoluble, polymeric form [4, 6]. If no catalyst is used, the hemicellulose is presumably solubilized as hemicellulose oligo- or polymers by the organic acids present in the native straw (i.e., by acetic acid and water itself) [7, 8].

The steam pretreatment in conjunction with an acid catalyst is known to release the hemicellulose constituents of lignocellulose as oligo- and monosaccharides; however, during the heat treatment some of the released monosaccharides may be degraded to compounds inhibitory to both cellulase enzymes and the yeast during the subsequent fermentation step [9, 10]. These inhibitory compounds include weak acids, furfural (from xylose) and 5-hydroxymethyl furfural (from C6 monosaccharides), and phenolic compounds from lignin [4, 9]. Hot liquid water extraction is a particularly attractive pretreatment process compared to steam explosion as this pretreatment involves no handling of harsh chemicals. Moreover, hot liquid water extraction is reported to produce a liquid stream, which, in contrast to acid hydrolyzed steam pretreated lignocellulose, does not inhibit the yeast during the fermentation step as hemicellulose is mainly released as oligomers [7]. Evidently, the specific pretreatment process best suited to a specific process will depend on a number of factors, including the origin of the lignocellulosic biomass—softwood, hardwood, herbaceous energy crops or other agricultural residues—and the amount and nature of inhibitory compounds. The choice of pretreatment also depends on whether the C5 monosaccharides are supposed to be utilized or not for the bioethanol production [8, 9].

Despite the intensive experimental investigation of different pretreatment methods only very limited efforts have been devoted to systematically compare the influence of different pretreatment strategies on the enzymatic cellulose hydrolysis of wheat and barley straw substrates. In this work, we have compared the influence of three principally different pretreatment strategies on the subsequent enzymatic hydrolysis of wheat and barley straw biomass. The glucose levels and relative yields obtained were compared after cellulytic
enzyme treatments with a commercial cellulase product Celluclast 1.5 L® from *Trichoderma reesei* supplemented with β-glucosidase, Novozyme 188, from *Aspergillus niger*.

**Materials and Methods**

**Substrate**

Barley and wheat straw were grown and harvested in 2006 on the island of Funen, Denmark. Samples of both types of straw were transported to DONG Energy (Danish Oil and Natural Gas Energy), Denmark and to the Department of Chemical Engineering, Lund University, Sweden (Professor G. Zacchi) for pretreatment. The specific conditions of the different pretreatments are specified in Table 1. In brief; the DONG Energy pretreatment method consisted of a three-stage process, which involved triple heating treatment of the straw at increasing temperatures. After the first heating step, the liquids were removed. At Lund University, the barley and wheat straw were subjected to steam explosion, both with and without the presence of an acid catalyst (in this case H₂SO₄). A major difference between the pretreatment procedures was the scale of processing; at DONG Energy the straw was fed directly to the reactor in whole bales, whereas at Lund University, the straw was chopped into pieces, approximately 2 cm long, before being fed to the reactor in batches of 400 g.

**Enzymes**

The enzyme system applied consisted of Celluclast 1.5 L® (Celluclast) derived from *T. reesei*, and Novozyme 188 (NS 188) derived from *Aspergillus niger*; both preparations were from Novozymes A/S (Bagsvaerd, Denmark). The activity of the Celluclast preparation was 47 FPU g⁻¹ (FPU=filter paper units) as measured by the standardized filter paper assay provided by the US National Renewable Energy Laboratory [11]. The protein content of the Celluclast preparation was measured using a Micro BCA™ Protein Assay Kit (Pierce, Rockford, IL) following the manufacturer’s instructions; before the

<table>
<thead>
<tr>
<th>Code</th>
<th>Substrate</th>
<th>Pretreatment conditions</th>
<th>Dry matter % w/w</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley 1</td>
<td>Acid-impregnated, steam-exploded barley straw</td>
<td>60% DM, 1% H₂SO₄, 1 h; 220°C, 5 min</td>
<td>19±0.3</td>
</tr>
<tr>
<td>Barley 2</td>
<td>Water-impregnated, steam-exploded barley straw</td>
<td>70% DM, 1 h; 210°C, 5 min</td>
<td>20±1.5</td>
</tr>
<tr>
<td>Wheat 1</td>
<td>Acid-impregnated, steam-exploded wheat straw</td>
<td>60% DM, 0.2% H₂SO₄, 1 h; 190°C, 10 min</td>
<td>18±2.0</td>
</tr>
<tr>
<td>Wheat 2</td>
<td>Water-impregnated, steam-exploded wheat straw</td>
<td>70% DM, 1 h, 220°C, 2.5 min</td>
<td>26±1.2</td>
</tr>
<tr>
<td>Barley 3</td>
<td>Hot-water-extracted barley straw</td>
<td>Approx. 16% DM, 60°C, 15 min; liquids removed; 180°C, 10 min; 195°C, 3 min</td>
<td>24±1.0</td>
</tr>
<tr>
<td>Wheat 3</td>
<td>Hot-water-extracted wheat straw</td>
<td>Approx. 16% DM, 60°C, 15 min; liquids removed; 180°C, 10 min; 195°C, 3 min</td>
<td>28±0.6</td>
</tr>
</tbody>
</table>

*Pretreatment was done at optimal conditions according to [10] and [13]*
protein determination the Celluclast was desalted using a Micro Bio-Spin® 6 Chromatography Column (Bio-Rad, Hercules, CA) eluting with 0.05 M sodium acetate buffer pH 5. The activity of NS 188 was 260 CBU g\(^{-1}\) (Cellobiase Units) determined from glucose production on cellobiose at 40°C, pH 5 (provided by Novozymes A/S, Bagsvaerd, Denmark).

Hydrolysis Reactions

The hydrolytic enzyme treatment reactions were carried out at 5 FPU Celluclast per gram dry matter (DM) supplemented with 13 CBU of NS 188. The enzyme reactions were carried out in 100 g scale in a shaking incubator (New Brunswick, Innova 44, Edison, NJ) at 200 RPM. The various substrates were weighed and buffer (0.5 M sodium acetate, pH 5) was added to adjust the substrate DM content in the reactions to 10.0% w/w DM. Samples were drawn from the reaction at specified time points, boiled for 10 min to halt the enzyme reactions, and centrifuged for 10 min at 14,000×g. Each supernatant was then collected for monosaccharide analysis by high-performance anionic exchange chromatography (HPAEC), see below. The deactivation of the Celluclast and NS 188 enzyme system during reactions was tested by incubating the enzyme preparations in buffer at 50°C for 72 h corresponding to the reactions on the substrates. After the incubation, the remaining activity was measured using the standardized filter paper assay [11].

Analysis of Substrate Monosaccharides and Glucose Yields

The glucose, arabinose, and xylose (and galactose) concentrations were analyzed by HPAEC on a Dionex® BioLC system equipped with a CarboPac PA1 column (4×250 mm) (Dionex Denmark A/S, Hvidovre, DK) and a CarboPac PA1 guard column (4×50 mm). Samples were eluted isocratically with 0.01 M KOH at a flow rate of 1 ml/min and analytes were detected and quantified against standard curves by electrochemical detection in a pulsed amperometric detection mode as described previously [12].

Analysis of Oligomer Profiles by Matrix-assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) was performed on supernatant fractions of pretreated straw in the positive ion mode on a Voyager DE STR mass spectrometer (Perseptive Biosystems, Framingham, MA, USA). Analyses were carried out in the reflector mode at a mass range of 500–4,000 m/z with an accelerating voltage of 20 kV and a delay time of 400 ns. A low mass gate value of m/z 500 was selected for analysis to avoid saturation of the detector; 20 mg ml\(^{-1}\) DHB in 70% ACN/0.1% TFA (w/w) was used as matrix. The samples were subjected to ion exchange (AG 50W-x12 analytical grade cation exchange resin 100–200 mesh hydrogen, Bio-Rad [Hercules, CA]) before being applied to the matrix.

Results

Glucose Potential After Pretreatment

To assess the glucose potential and evaluate the hemicellulose monosaccharides in the differently pretreated batches of barley and wheat straw, the monosaccharide composition in...
each of the substrates was analyzed before enzymatic treatment. Based on the measured levels of arabinose and xylose (only negligible amounts of galactose were detected; Table 2) the hemicellulose was estimated to make up approximately 5–12% of the dry matter—and, as expected, to be mostly contributed by xylan. The glucose potential was defined as the amount of glucose available after acid hydrolysis of the pretreated lignocellulose (Table 2). Except for the hot-water-extracted samples (Barley 3 and Wheat 3), the pretreated barley straw samples had a higher glucose potential than the pretreated wheat straw. The glucose potentials of the acid- or water-impregnated, steam-explored barley straw samples ranged from 57 to 61% of the DM, whereas the equivalent values for the wheat straw samples were 50–55% (Table 2). For both wheat and barley straw, the DONG Energy hot water extraction process resulted in a lower glucose potential (41–48% of the DM) than the steam explosion pretreatment (Table 2).

The pretreatment conditions used for the steam explosion pretreatments were carefully selected as optimal for each of the substrates [10, 13]. Hence, the acid-impregnated, steam-explored barley straw pretreatment was somewhat harsher than the equivalent treatment for the wheat straw with respect to the amount of H₂SO₄ and the temperature during treatment (Table 1). The observed higher glucose potential in the barley straw compared to the wheat straw could be caused by the slightly harsher pretreatment conditions employed for the barley straw. Alternatively, the levels could be a result of barley straw simply being richer than wheat straw in cellulose per unit dry matter. The composition of straw is dependent on the straw variety and the climatic factors during the growth season. Whereas the hemicellulose levels have been found to be approximately similar, the cellulose content has consistently been found to be a little higher in barley straw than in wheat straw—wheat straw is thus slightly more lignified and also contains more silica than barley straw [14].

### Enzymatic Glucose Release

The enzymatic hydrolysis reactions were run for 72 h; samples were taken at time points 2, 4, 6, 24, 48, and 72 h. However, as the structure of the substrate was not degraded to any substantial degree until after 24 h of reaction—as evaluated from the glucose levels released—only the results originating from samples taken at 24 and 72 h are considered in the following. The pretreatment processes evaluated in this study could be ranked according to severity; H₂SO₄ impregnation followed by steam explosion was considered the harshest pretreatment in terms of breaking the lignocellulose structure. In the next place came the water impregnation with steam explosion and finally the liquid hot water extraction, which was considered the least harsh pretreatment method. According to the severity of the pretreatment, the released glucose levels, in the case of barley straw, were highest with the most severe pretreatment and lowest with the liquid hot water extraction (Fig. 1).

### Table 2  Carbohydrate composition of straw substrates: values are given in percent of total dry matter.

<table>
<thead>
<tr>
<th></th>
<th>Arabinose</th>
<th>Xylose</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley 1</td>
<td>5.4±0.1</td>
<td>5.5±0.4</td>
<td>57±7.4</td>
</tr>
<tr>
<td>Barley 2</td>
<td>4.4±1.5</td>
<td>5.3±0.8</td>
<td>50±0.3</td>
</tr>
<tr>
<td>Barley 3</td>
<td>2.2±0.1</td>
<td>5.8±0.1</td>
<td>55±7.1</td>
</tr>
<tr>
<td>Wheat 1</td>
<td>4.4±1.5</td>
<td>5.3±0.8</td>
<td>50±0.3</td>
</tr>
<tr>
<td>Wheat 2</td>
<td>2.2±0.1</td>
<td>5.8±0.1</td>
<td>55±7.1</td>
</tr>
<tr>
<td>Wheat 3</td>
<td>5.8±1.0</td>
<td>7.1±0.6</td>
<td>48±7.8</td>
</tr>
</tbody>
</table>
However, the final glucose levels obtained from the barley straw subjected to steam explosion pretreatment with either water or H$_2$SO$_4$ impregnation did not differ substantially after 72 h of reaction (Fig. 1, Barley 1 versus 2). The enzymatic treatment of the hot-water-pretreated barley straw (Barley 3) resulted in a glucose concentration that tended to be lower than that obtained by steam explosion, suggesting that the steam explosion pretreatment rendered the barley straw more easily degradable. However, when comparing the glucose concentrations in relation to the amount of potential glucose in the residues (yield percent: [g glucose × g potential glucose$^{-1}$] × 100%), the hot-water-extracted straw appeared to be more efficiently enzymatically hydrolyzed than the steam pretreated substrates (Fig. 1).

With regard to glucose levels obtained, the trends for wheat were opposite those of barley. Hence, for the wheat straw the highest glucose levels were achieved with the least severe pretreatment, namely, the hot water extraction pretreatment—the glucose concentrations obtained on the pretreated wheat samples did not, however, differ significantly after the comparative enzymatic hydrolysis (Fig. 2). However, the yield obtained in the enzymatic hydrolysis of Wheat 3 (after 72 h) was 10–15% higher than the yields obtained in the other enzymatic hydrolysis reactions with Wheat 1 and 2 (Fig. 2). In all three enzymatic hydrolysis reactions with wheat straw, the glucose concentrations and the yields were lower than those obtained with barley straw (Fig. 1 versus 2). These results might be related to the interplay between the differences in straw structure of barley and wheat and the difference in pretreatment conditions as the least severe pretreatment condition gave the highest glucose concentration and yield for wheat straw, whereas the opposite was true for barley straw. Taken together, the data were in accordance, but expanded, to previous findings [10], indicating that barley and wheat straw differ with respect to the conditions needed for efficient opening of the lignocellulose structure for enzymatic hydrolysis.

Supplementation of Enzymes During Reaction

Cellulases acting on pretreated straw may lose activity if adsorbed to the lignin or the crystalline cellulose in the substrate; alternatively, the enzymes may be thermally inactivated during the reaction or inhibited by substances resulting from the pretreatment. In all cases, the enzymatic hydrolysis, and in turn the glucose release, will be lower than optimal. To assess whether any of these effects could be overcome in practice by addition of more enzymes during hydrolysis, an experiment was carried out in which extra enzymes (in the form of Celluclast + NS 188) were added to the hydrolysis reactions after 24 h of reaction. The released glucose levels obtained with this addition of extra enzymes after 72 h were highest for the hydrolysis of barley straw and especially high for the barley straw subjected to acid pretreatment.
pretreatment, Barley 1, which gave a maximal concentration of released glucose of 39 g L\(^{-1}\) after 72 h (Table 3). The reaction included 48 h of reaction with additional enzymes as the extra enzymes were added at 24 h. After a total reaction time of 72 h the boosting effect was equivalent to a relative glucose increase with extra enzymes of \(\sim 54\%\) as compared to the nonsupplemented reaction (\([39.2-25.4] \times 100)/25.4 \approx 54\%\), Table 3) and a relative increase in glucose concentration of 89\% from 24–72 h with the extra enzymes added (Table 3). This relative increase should be compared to a relative increase in glucose concentration from 24–72 h of \(\sim 23\%\) during the regular enzymatic hydrolysis of Barley 1 (Table 3).

The highest relative increases in released glucose levels by addition of extra enzymes were obtained for the pretreated wheat samples. For Wheat 1 and Wheat 2 the extra enzyme addition boosted the relative increase in glucose concentrations by \(\sim 180–200\%\) during the reaction from 24 to 72 h (Table 3). Addition of extra enzymes after 24 h of reaction thus significantly increased the yields with all the four tested pretreated substrates (Table 3).

It is well documented that adsorption of enzymes to both crystalline cellulose and lignin may significantly decrease the rate and extent of cellulolytic degradation of cellulose in lignocellulosic substrates [15, 16]. Moreover, inhibitors generated during steam explosion pretreatment may exert a negative impact on the enzymatic hydrolysis of the straw [17]. In the pretreated Barley 1 and 2 and in the Wheat 1 and 2 samples, both furfural and 5-

![Fig. 2 Glucose concentration (columns) and yield (black diamonds) (based on the potential glucose in the substrate as determined by acid hydrolysis) after 72 h hydrolysis of pretreated wheat straw at 10% w/w dry matter. Legends refer to pretreatment conditions shown in Table 1.](image-url)

**Table 3** Released glucose levels (g L\(^{-1}\)) and relative increases in percent after 24 and 72 h of enzyme treatment (Celluclast + Novozyme 188) without and with supplementation of extra Celluclast + Novozyme 188 after 24 h of reaction.

<table>
<thead>
<tr>
<th></th>
<th>Glucose release (g L(^{-1}))</th>
<th>24 h</th>
<th>72 h</th>
<th>72 h extra enzyme(^a)</th>
<th>24–72 h % increase</th>
<th>24–72 h % increase extra enzyme(^b)</th>
<th>72 h % relative improvement with extra enzyme(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley 1</td>
<td></td>
<td>20.7±4</td>
<td>25.4±2</td>
<td>39.2±3</td>
<td>22.7</td>
<td>89.4</td>
<td>54.3</td>
</tr>
<tr>
<td>Barley 2</td>
<td></td>
<td>15.9±2</td>
<td>24.6±3</td>
<td>32.0±1</td>
<td>54.7</td>
<td>101</td>
<td>30.1</td>
</tr>
<tr>
<td>Wheat 1</td>
<td></td>
<td>9.6±4</td>
<td>18.5±4</td>
<td>26.9±4</td>
<td>92.7</td>
<td>180</td>
<td>45.4</td>
</tr>
<tr>
<td>Wheat 2</td>
<td></td>
<td>9.8±1</td>
<td>19.5±4</td>
<td>29.3±4</td>
<td>99.0</td>
<td>199</td>
<td>50.3</td>
</tr>
</tbody>
</table>

\(^a\)Extra dose of Celluclast and Novozyme 188 added after 24 hours of reaction

\(^b\)Each value calculated as the difference in percent between the glucose concentration after 24 h and 72 h of enzyme reaction

\(^c\)Each value calculated from relative difference in the glucose release (g L\(^{-1}\)) at 72 h between samples with extra enzyme added and samples without extra enzyme added
hydroxymethyl furfural were identified by HPLC analysis of the (non-enzyme treated) supernatants (data not shown)—but the differences in the levels of these substances could not explain the different yields obtained after enzymatic hydrolysis. Also other mechanisms, such as the cellulases getting “stuck” within the cellulose, may slow down the enzymatic hydrolysis during cellulolytic hydrolysis reactions [18]. Another limiting factor may be that the enzymes are simply heat inactivated during the prolonged hydrolysis reaction at 50°C. To test this latter hypothesis, we measured the remaining FPU activity of the enzymes (Celluclast and NS188 incubated together) after 72 h of incubation in buffer at pH 5, 50°C. This resulted in a loss of approximately 80% of the initial enzyme activity (FPU) after 72 h of reaction (data not shown). Hence, although the presence of substrate may partly stabilize the enzymes, the gradual slowing down of the hydrolysis rates observed during prolonged enzymatic treatment of lignocellulosic biomass may partly be a result of gradual heat inactivation of the added cellulases—in addition to adsorption of enzymes to lignin and cellulose. This finding provides a strong stimulus to develop more heat-stable cellulases for hydrolysis of lignocellulosic biomass.

Oligomer Profiles of Supernatants

To fully evaluate the differences between the differently pretreated biomass samples, the oligomeric profiles of the supernatants of the pretreated Barley 1, Barley 2, Wheat 1, and Wheat 2 substrates were examined by Maldi-TOF MS analysis. As it was not possible to remove any liquids from Barley 3 and Wheat 3, the liquid fractions in this material were not analyzed. The oligomeric profiles obtained by the MS analysis differed significantly in accordance with the type of straw and in response to the use of acid or water impregnation before the steam explosion pretreatment. The supernatant of the acid-impregnated and steam-exploded barley and wheat straw substrates, Barley 1 and Wheat 1, both mainly contained hexose oligomers ranging from 5 C₆ (HO5) up to 16 C₆ (HO16) (Fig. 3a,c). In addition, the Barley 1 and Wheat 1 supernatants also harbored a few, relatively short pentose oligomers: In the Barley 1 supernatant, the analyzed pentose oligomers were a mixture of hexa-, hepta-, and octamers (PO6-PO8, Fig. 3a), whereas in the Wheat 1 supernatant, the pentose oligomers were penta- and hexa-oligomers, and included acetylated pentamer species (PO5, PO6, PO5AC, Fig. 3c). In comparison, the water-impregnated supernatants from Barley 2 and Wheat 2 both contained a much wider profile of pentose oligomers containing from three to four and up to 13 C₅ monomers (Fig. 3b,d). In both of these supernatants, the pentose oligomers were found to occur with and without acetic and ferulic acid substitutions. The water-impregnated and steam-exploded barley supernatant, Barley 2, moreover appeared to contain a wide range of hexose oligomers (HO5-HO14, Fig. 3b). These were not found in the corresponding water-impregnated supernatant from wheat straw, Wheat 2, which appeared to only harbor pentose oligomers (Fig. 3d).

The Maldi-TOF MS chromatograms thus revealed significant differences of the impact of the acid vs. water impregnation pretreatment on the oligomer profiles on the differently pretreated barley and wheat straw. These differences between acid and water impregnations before the steam explosion confirmed that the hemicellulose is almost completely solubilized to monomeric species (not detected in the MS analysis) in acid-impregnated samples, whereas the hemicellulose is apparently solubilized in the form of a wider spectrum of oligomers with water impregnation and steam explosion. The results also indicated significant differences in the hexose oligomer structures between barley and wheat straw after pretreatments employing water impregnation and steam explosion. The currently
Fig. 3 MALDI-TOF MS spectra (sodium adducts) of the liquid fractions from Barley 1 (a), Barley 2 (b), Wheat 1 (c), and Wheat 2 (d), respectively. HO denotes hexose oligomer, PO denotes pentose oligomer, FeA denotes ferulic acid and Ac denotes acetic acid. Numbers refer to the number of monosaccharides in the oligomer.
Fig. 3 (continued)
available knowledge does not provide a clear explanation for this difference—other than the biological difference between barley and wheat straw. The wheat straw pretreatment conditions included treatment at a slightly higher temperature by only 10°C, but on the other hand, a shorter treatment time of only 2.5 min (Table 1). As the Maldi-TOF MS chromatograms give only qualitative, and not quantitative, oligomeric profiles, the potential glucose and xylose yields contributed by the oligomers in the supernatants could only be compared after enzymatic hydrolysis of these four supernatants. The glucose concentration, including the net gain contributed by enzymatic hydrolysis of the oligomers, was highest for both of the barley supernatants (Fig. 4). However, hydrolysis of the hexose oligomers in the supernatant(s) contributed a gain of only maximum ~2 g glucose L\(^{-1}\) (Fig. 4). The lowest contribution, found with Wheat 2, was only marginal: ~0.5 g glucose L\(^{-1}\) (Fig. 4). This latter finding was in accordance with the MS profile, which showed that the Wheat 2 supernatant did not contain detectable amounts of hexose oligomers. On the other hand, the supernatant of the latter harbored the highest pentose (xylose) potential: thus, the xylose released by enzymatic hydrolysis, the pentose oligomers brought about by the Celluclast+NS 188 enzyme treatment of the Wheat 2 supernatant, was ~7 g xylose L\(^{-1}\) (Fig. 4). The net xylose yields from the other supernatants varied from 2 to 6 g xylose L\(^{-1}\), and was lowest for the Barley 1 supernatant (Fig. 4), which, on the other hand, had the highest xylose content before the addition of enzymes. These results confirmed that the acid impregnation and steam explosion directly released most of the xylose from both the barley and wheat straw substrates.

Effect of Substrate Loading Level

The effect of substrate loading was of interest, as it has previously been shown that increasing the substrate concentration results in higher glucose concentration but lower overall yields [19, 20]. The substrate that resulted in the highest glucose concentration after enzymatic hydrolysis was the acid treated, steam exploded barley straw, Barley 1 (Table 3). Hence, the effect of lowering the substrate concentration was evaluated for this substrate to assess if it was possible to obtain a higher yield ([g glucose \times g potential glucose\(^{-1}\)] \times 100\%) with lower substrate loading. As expected, a reduction of the substrate loading from 10% w/w to 2.5% w/w increased the final extent of conversion as evaluated from the yields of glucose, but resulted in a lowered released glucose concentration (Fig. 5). A compromise between maximum yield versus maximum glucose concentration after enzymatic hydrolysis was found at the substrate dry matter concentration of 5.0% w/w (Fig. 5). This substrate loading resulted in a glucose concentration of approximately 20 g L\(^{-1}\) and a yield of ~67% of the available glucan in the straw (Fig. 5). The occurrence of a clear positive relation between the final glucose...

**Fig. 4** Xylose and glucose concentrations measured in the liquid fractions of the pretreated straw samples. Black and dark gray correspond to xylose concentration before and after incubation with Celluclast + NS188, respectively. Light gray and white correspond to glucose before and after addition of Celluclast + NS 188, respectively.
concentration and the DM substrate level, and a negative relation between glucose yields and substrate DM concentration were in complete accordance with previous data [19, 20].

Conclusions

The highest glucose release was found after enzymatic hydrolysis of barley straw, which had been pretreated using H₂SO₄ impregnation and steam explosion. Ranked after this was the barley straw subjected to water impregnation and steam explosion pretreatment. The lowest enzyme-catalyzed glucose release from barley straw was obtained for the substrate having been subjected to hot water extraction pretreatment. An opposite trend of pretreatment efficacy was found for wheat straw. These results indicate that the optimal pretreatment conditions differ substantially for wheat and barley straw with respect to the resulting cellulose substrate accessibility and in turn with respect to glucose concentration to be obtained by enzymatic hydrolysis. It was also shown that addition of extra cellulolytic enzymes to the hydrolysis reaction had a significantly positive impact on glucose release when the enzymes were added after 24 h reaction. Addition of the extra enzymes gave a maximal glucose release of 39 g L⁻¹ for the barley straw, which had been acid-impregnated before steam explosion, but the relative increase in glucose liberation by addition of more enzymes during the reaction versus the regularly enzyme-treated samples were largest for the wheat samples irrespective of their pretreatment. It was also shown that the cellulolytic enzymes (Celluclast + NS 188) lost significant activity as a result of thermal inactivation during prolonged incubation at 50°C. These results indicated that some of the problems of decreased glucose liberation during prolonged enzymatic lignocellulose hydrolysis might be overcome by simply adding more enzyme during the reaction. The cost–benefit analysis between the cost investment effectuated by addition of extra enzymes to achieve higher glucose levels and higher ethanol yields versus the revenues obtained from the extra ethanol yields awaits further study.

Acknowledgments

This research was partially financed by the European Commission Framework V, contract no. NE5/2001/685 (The Babilafuente Bioethanol Project). Danish Oil and Natural Gas Energy, Guido Zacchi and Marie Linde, Lund University, Sweden, are thanked for their assistance in pretreating the barley and wheat straw. We also thank Carsten P. Sönksen Novozymes A/S for access to MALDI-TOF MS equipment.

Fig. 5 Glucose concentrations (filled squares) and yields in percent of substrate glucan (open squares) from Barley 1 after 72 h of enzymatic treatment (Celluclast + NS 188) as a function of dry matter. The yields were calculated as percent of glucose obtained in relation to the potential glucose release possible from the native barley straw ([g glucose×g potential glucose⁻¹]×100%).
References

Effects of Substrate Loading on Enzymatic Hydrolysis and Viscosity of Pretreated Barley Straw

Lisa Rosgaard · Pavle Andric · Kim Dam-Johansen · Sven Pedersen · Anne S. Meyer

Abstract In this study, the applicability of a “fed-batch” strategy, that is, sequential loading of substrate or substrate plus enzymes during enzymatic hydrolysis was evaluated for hydrolysis of steam-pretreated barley straw. The specific aims were to achieve hydrolysis of high substrate levels, low viscosity during hydrolysis, and high glucose concentrations. An enzyme system comprising Celluclast and Novozyme 188, a commercial cellulase product derived from Trichoderma reesei and a β-glucosidase derived from Aspergillus niger, respectively, was used for the enzymatic hydrolysis. The highest final glucose concentration, 78 g/l, after 72 h of reaction, was obtained with an initial, full substrate loading of 15% dry matter weight/weight (w/w DM). Conversely, the glucose yields, in grams per gram of DM, were highest at lower substrate concentrations, with the highest glucose yield being 0.53 g/g DM for the reaction with a substrate loading of 5% w/w DM after 72 h. The reactions subjected to gradual loading of substrate or substrate plus enzymes to increase the substrate levels from 5 to 15% w/w DM, consistently provided lower concentrations of glucose after 72 h of reaction; however, the initial rates of conversion varied in the different reactions. Rapid cellulose degradation was accompanied by rapid decreases in viscosity before addition of extra substrate, but when extra substrate or substrate plus enzymes were added, the viscosities of the slurries increased and the hydrolytic efficiencies decreased temporarily.

Keywords Lignocellulose · Enzymatic hydrolysis · Glucose yield · Viscosity

Introduction

Starch and sucrose stocks are well-established raw materials for industrial ethanol manufacture that provide alcohol for alcoholic beverages, various technical purposes,
and fuel ethanol for blending with gasoline [1, 2]. For many years attention has been focused on also utilizing lignocellulosic biomass, e.g., straw, softwood, hardwood chips, and corn stover for industrial production of ethanol for fuel purposes [3, 4]. One of the prerequisites for the efficient utilization of lignocellulose for the production of ethanol is to produce a fermentable hydrolysate with a sufficiently high glucose concentration to provide a feasible ethanol concentration for the subsequent distillation. To obtain this high glucose concentration, a sufficiently high lignocellulose substrate loading is required. However, because of the high viscosity of most lignocellulosic substrates, it is difficult to operate at solids loadings much higher than approximately 10% by weight of lignocellulose concentrations [5]. The high viscosity is mainly because of the presence of relatively high contents of insoluble materials, but also a result of the high water-binding capacity of the hemi- and lignocellulose in the substrate material. Most lignocellulosic residues must undergo a pretreatment process to remove portions of the lignin and hemicellulose and increase porosity, thereby improving the accessibility of the substrate to cellulases [6]. Although the cellulose hydrolysis can be accomplished by acid treatment, employment of enzymes for the hydrolysis is considered as a prospectively more viable strategy, notably to provide a more environmentally friendly process, but also to avoid generation of byproducts that may inhibit the subsequent fermentation of glucose to ethanol [7, 8].

When it is considered that current starch-based processes are conducted at substrate loadings as high as 33–37% dry matter weight/weight (w/w DM) [9], the requirement for high substrate dry matter (DM) loadings pose a particular challenge for the enzymatic conversion of lignocellulosic substrates. Empirically, it is known that the viscosity of the lignocellulosic substrates decreases as a result of cellulolytic activity, presumably because the lignocellulose looses its structure and water-binding capacity upon cellulose degradation. However, the initially high viscosity of (pretreated) lignocellulosic materials currently prevents efficient mixing. Moreover, the unproductive binding of cellulases to the lignin, as demonstrated for Trichoderma reesei-derived cellulases [10], may increase at high substrate loadings, especially as the hydrolysis proceeds and the amount of cellulose decreases. A way to increase the solids loading for the enzymatic hydrolysis might be to add multiple batches of substrate to overcome the extremely high initial viscosity, which hinders blending and slows hydrolysis. A secondary aim, notably for industrial processing, would be to retain a relatively low viscosity throughout hydrolysis, and thus obtain conversion of high substrate levels to achieve the desired high glucose levels.

Previously, such a fed-batch strategy was demonstrated to work relatively successfully in simultaneous saccharification and fermentation (SSF) on wet oxidized corn stover obtaining final ethanol levels of 8vol.% with substrate concentrations of 15% w/w DM [5]. Likewise, addition of fresh substrate to partially hydrolyzed steam-pretreated spruce was previously shown to result in a boost in glucose release during enzyme hydrolysis with purified cellulbiohydrolase I and endoglucanase II [11]. However, the effect of sequential substrate loading in relation to viscosity and cellulose conversion of steam-pretreated straw with an industrially relevant cellulase plus β-glucosidase system has not been investigated.

This report presents an evaluation of a substrate fed-batch strategy to increase the loading of steam-pretreated barley straw during enzymatic hydrolysis at 50 °C. The strategy has been evaluated with respect to cellulose conversion and decrease in viscosity during hydrolysis with the aim of providing some insight into the interplay between substrate loading, viscosity, and enzyme-catalyzed cellulose degradation of the straw material.
Methods

Substrate

Steam-pretreated barley straw was provided by Professor Guido Zacchi at the Department of Chemical Engineering, Lund University, Sweden. The straw had been soaked in 1% w/w sulfuric acid and then steam-pretreated at 170 °C for 5 min. After this pretreatment the slurry had a DM content of 15% w/w. Immediately after the pretreatment procedure, the slurry was frozen (−20 °C) in aliquots and transported to Denmark, where it was kept frozen until use. To bring the DM content up to 21% w/w, gently thawed slurry samples were pooled and vacuum filtered through a 1.6-μm glass fiber filter GA-55 (Frisinette Aps, Knebel, Denmark). The DM content was then determined by overnight drying of substrate samples at 105 °C. The determination of structural carbohydrates composition and Klason lignin contents were determined according to the US National Renewable Energy Laboratory (NREL) standard [12]. For the enzymatic hydrolysis, the substrate was weighed and buffer was added (1 M of citric acid monohydrate, pH 5) to the desired percent by weight of DM.

Enzymatic Hydrolysis

The enzyme system applied consisted of Celluclast 1.5L® and Novozyme 188 (NS 188) (Novozymes A/S, Bagsvaerd, Denmark). The filter paper activity of Celluclast was 47 filter paper units (FPU)/g according to the standardized filter paper assay from NREL [13]. The activity of NS 188, derived from Aspergillus niger, was 246 cellobiase units (CBU)/g. The CBU activity was determined by measuring glucose production on cellobiose at 40 °C, pH 5 (provided by Novozymes A/S). For the hydrolytic reactions the enzyme loading was 7.5 FPU/g DM of Celluclast and 13 CBU/g DM of NS 188. The enzymes were dosed based on the final total amount of DM loaded into the reaction. Three reactions with fixed substrate loadings of 5, 10, and 15% w/w DM were done. Two reactions starting at 5 and 10% w/w DM were supplied with additional substrate after 6 and 24 h (“5+5+5%”) and 24 h (“10+5%”), respectively, to increase the substrate loading to a final 15% w/w DM. Accordingly, these reactions had a higher enzyme to substrate ratio in the first 24 h compared to reactions with constant substrate loading (as specified in Table 1). Two comparable reactions were carried out with simultaneous addition of extra substrate plus

<table>
<thead>
<tr>
<th>Substrate addition mode</th>
<th>E/S % ((g enzyme/g DM)×100)</th>
<th>E conc. % ((g enzyme/g liquids)×100)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0–6 h</td>
<td>6–24 h</td>
</tr>
<tr>
<td>5+5+5%</td>
<td>255</td>
<td>85.3</td>
</tr>
<tr>
<td>10+5%</td>
<td>73.8</td>
<td>73.8</td>
</tr>
<tr>
<td>5%+(5%+E)+(5%+E)</td>
<td>25.7</td>
<td>25.7</td>
</tr>
<tr>
<td>10%+(5%+E)</td>
<td>25.7</td>
<td>25.7</td>
</tr>
<tr>
<td>5%</td>
<td>25.7</td>
<td>25.7</td>
</tr>
<tr>
<td>10%</td>
<td>25.7</td>
<td>25.7</td>
</tr>
<tr>
<td>15%</td>
<td>25.7</td>
<td>25.7</td>
</tr>
</tbody>
</table>
extra enzyme (denoted “5%+[5%+E]+[5%+E]” and “10%+[5%+E]”); in these reactions enzyme and substrate were dosed to maintain a constant enzyme/substrate ratio similar to the reactions, which where kept at constant substrate loading (Table 1). The pH of substrate and buffer (1 M of citric acid monohydrate, pH 5) was adjusted to 5 before enzyme addition with 1 M of NaOH and readjusted in the cases where additional substrate was added.

The individual hydrolysis reactions were carried out in 2-l bottles in a thermostated water bath (50 °C) placed in a chamber fitted with a motor (RW 47D from Janke and Kunkel, GmbH. & Co. Staufen, Germany) able to drive six identical, custom-made impellers (Janke and Kunkel, GmbH. & Co.). Each impeller had three sets of three-winged blades, which were distributed along a rod connecting to the motor running at 57 rpm. During hydrolysis, the samples were taken at specific time points and enzyme activity was terminated by boiling each sample for 10 min. Each sample was then centrifuged (14,000 rpm for 10 min) and the supernatants were collected for glucose analysis (see below).

Analysis of Substrate Monosaccharides and Glucose Yields

Glucose, xylose, arabinose, and galactose were separated by high-performance anionic exchange chromatography on a Dionex® BioLC system equipped with a CarboPac PA1 column (4×250 mm) (Dionex Denmark A/S, Hvidovre, Denmark) and a CarboPac Pa1 guard column (4×50 mm). Samples were eluted isocratically with 0.01 M of KOH at a flow rate of 1 ml/min and analytes were detected and quantified against standard curves by electrochemical detection in a pulsed amperiometric detection mode as described previously [14]. Significant differences between glucose concentrations were established with a pooled standard deviation of 3.6 using the Minitab 12.11 software (Minitab Inc., Addison-Wesley, Reading, MA, USA).

Rheometer Techniques

Apparent viscosities of slurries were measured at specific time points during enzymatic hydrolysis by means of a DV-III Ultra rheometer with a full-scale spring torque of 7187 dyne/cm, equipped with a vane spindle (no. 72), and controlled by the Rheocalc© program (all from Brookfield Engineering Lab. Inc., VWR International, Roedovre, Denmark). Measurements were preferably taken at torque readings between 10 and 90% of the full-scale spring torque. This range corresponded to a shear rate range of 4.6–51.43/s. For each time point viscosity data were collected every second, for 20 s, starting at 20 rpm, increasing to 220 rpm with speed increments of 10 rpm. The shear rate, \( \gamma \), was calculated by the following equation:

\[
\gamma = \frac{2 \cdot \omega \cdot R_c^2 \cdot R_b^2}{r^2 \cdot (R_c^2 - R_b^2)}
\]

where \( \omega \) is the angular velocity of the spindle (rad/s), \( R_c \) the radius of the container (cm), \( R_b \) the radius of the spindle (cm), and \( r \) the radius of the spindle (cm).

The power law (Eq. 2) was used to model the viscosity and shear rate:

\[
\tau = K_{pl} \gamma^n \Rightarrow \eta = K_{pl} \gamma^{n-1}
\]

where \( \tau \) is the shear stress, \( \gamma \) the shear rate, \( \eta \) the viscosity, \( K_{pl} \) the consistency index, and \( n \) the power law index.
Results and Discussion

Substrate Composition

To assess the compositional makeup of the pretreated barley straw, the monosaccharide composition and lignin content of this substrate was analyzed before enzymatic treatment. The main part of the substrate was made up of glucan and lignin, which constituted 58 and 30% w/w DM, respectively of the steam-pretreated barley straw (Table 2). Based on the measured levels of arabinose, xylose, and galactose (Table 2), the hemicellulose was estimated to make up approximately 15% by weight of the DM, and the hemicellulose was mostly contributed by xylan. The data confirmed that the barley straw was mainly made up of glucan (cellulose), lignin, and hemicelluloses. The levels obtained agree well with recently published data on the chemical composition of barley straw, indicating that glucans make up 37–40%, lignin 19–24%, and xylans and other hemicellulose components contribute 18–27% by weight of the straw [15]. The finding that the pretreatment involving acid-soaking and steam explosion enhanced the levels of glucans at the expense of xylans and other hemicellulose components are also in accordance with the current knowledge on the influence of acidic pretreatment on barley straw [15].

Sequential Addition of Substrate

Glucose Concentrations

The highest final glucose concentration of 78 g/l after 72 h of hydrolysis was obtained in the reaction having a constant substrate loading of 15% w/w DM from the start of the hydrolysis (Table 3). This final glucose concentration was significantly higher than those obtained in the corresponding reactions to which substrate had been added gradually to reach a final substrate loading of 15% w/w DM (Table 3). In contrast, after 72 h of reaction, the lowest final glucose levels of 28 and 47 g/l were obtained with the constant substrate loadings of 5% and 10% w/w DM, respectively (Table 3). After 72 h of reaction, the glucose levels obtained in the reactions, having been subjected to substrate addition, i.e., in the 5+5+5% and 10+5% reactions, respectively, were lower but similar at 63–66 g/l and there was no significant difference between the glucose concentrations of these reactions and those obtained in the analogous reactions where enzyme was loaded together with the substrate during the reactions (Table 3). However, during the first 6 h of reaction the glucose concentration of the 5+5+5% reaction was twice the concentration of the regular 5% w/w DM reaction. This finding was in accordance with the fact that the enzyme to substrate ratio during the initial period of the reaction was ~10 times higher in the sequentially loaded reaction kept at 5% w/w DM the whole time (Table 1). Both the 5+5+5% and 10+5% reactions had significantly higher glucose concentration after 6 and 24 h than the corresponding reaction with constant substrate loading (Table 3). The relatively low final glucose concentrations obtained in the
sequentially loaded reactions could be related to the dilution of both enzyme and glucose that occurred upon addition of more substrate, which had a 21% DM content (as opposed to introducing 100% dry substrate). Loading of 21% DM substrate as opposed 100% DM was chosen to avoid artifacts, resulting from drying of the substrate and to provide a more industrially relevant substrate loading strategy.

**Efficient Glucose Yields** If the glucose yields are compared on the basis of amount of DM in the reactions (g/g DM), the dilution effect, discussed above, is adjusted for. In this case the enzymatic hydrolysis of the 5% w/w DM reaction, giving of final glucose yield of 0.53 g/g DM after 72 h of reaction was found to be slightly more efficient than the 10 and 15% w/w DM reactions (Fig. 1). The finding that lowering the lignocellulose substrate concentration results in higher hydrolytic efficiency, as judged from the glucose yields (Fig. 1), are in complete agreement with our previously published results on enzymatic hydrolysis of pretreated barley straw [14] and is also coherent with cellulolytic efficiency of the Celluclast 1.5L® plus Novozyme 188 system on other lignocellulosic substrates such as steam-pretreated softwood [16]. Several mechanisms have been proposed to explain this phenomenon, including product inhibition and nonproductive adsorption of enzymes to both lignin and cellulose [10, 11], but the main cause of the decreased hydrolytic efficiency of cellulases with increased levels of lignocellulose is still uncertain. In general, the reactions, which were supplemented with extra substrate during the course of reaction, were particularly efficiently hydrolyzed during the first 6 h of the hydrolytic run where the enzyme to substrate ratio was high (Table 3, Fig. 1). In particular, the ~10 times higher initial enzyme/substrate ratio in the 5+5+5% reaction (Table 1) resulted in very high yields reaching ∼0.45 g glucose/g DM after the first 6 h of reaction (Fig. 1). However, when fresh substrate was added to the reactions at 6 h and 24 h, the glucose yields decreased and did not fully recover after the final 72 h reaction time (Fig. 1). The final difference in glucose yields among the conventional, constant substrate reactions, and the different substrate fed runs, is assumed to be because of the dilution effect of the enzyme upon the substrate addition (Table 1), coupled with insufficient reaction time of the enzymes on the freshly added substrate. Hence, although the glucose yields were initially high, and although the enzyme vs substrate concentration ended up being exactly the same in the sequentially loaded and the constant substrate reactions (Table 1), the effect of feeding in more substrate consistently resulted in lower overall glucose yields. At the time when the first batch of extra substrate was added to the reaction, the initial glucose yields from the initially loaded substrate were 0.44 and 0.48 g glucose/g DM for the 5+5+5% after 6 h and the 10+5%

### Table 3 Glucose concentration (g/l) obtained at specific time points during enzymatic hydrolysis.

<table>
<thead>
<tr>
<th>Substrate DM loading (%) w/w</th>
<th>6 h</th>
<th>24 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>5+5+5%</td>
<td>25±3.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>43±1.1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>63±4.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10+5%</td>
<td>31±0.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>44±7.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>66±1.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5%+(5%+E)+(5%+E)</td>
<td>15±0.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>49±5.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>62±7.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10%+(5%+E)</td>
<td>24±1.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>44±7.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>67±4.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5%</td>
<td>13±1.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>24±2.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>28±1.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>10%</td>
<td>22±0.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>41±0.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>47±3.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>15%</td>
<td>31±1.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>60±1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>78±5.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Different superscript letters (a–e) indicate significantly different glucose concentrations (p<0.05).

* For codes, please see “Methods.”
after 24 h, respectively (Fig. 1). With such yields, the available cellulose substrate must have had decreased to a large extent, hereby increasing the probability of nonproductive enzyme to lignin binding. A declining enzymatic hydrolysis rate with time has been reported widely in relation to hydrolysis of lignocellulosic substrates, and several hypotheses have been presented to explain this phenomenon. Recent data on rate of enzyme catalyzed conversion of avicel cellulose indicate that the drop off in reaction rate with reaction progress could not be attributed to changes in substrate reactivity, but were rather because of other factors such as structural obstacles retarding the enzymes’ activity and/or processivity [17]. Other recent data support that nonproductive adsorption of cellulases to lignin via hydrophobic bonding may be an important mechanism explaining the drop in reaction rate during extended conversion of lignocellulose [10, 11, 18]. Nevertheless, it seems uncertain whether addition of fresh (lignocellulose) substrate can stimulate the desorption of lignin-bound enzymes to increase cellulose hydrolysis rate.

To mimic an industrially relevant enzymatic hydrolysis strategy, we employed the acidified, steam-pretreated barley straw substrate directly in the reactions, albeit the reaction pH was always adjusted for the hydrolysis to proceed. In the study by Eriksson et al. [11], where addition of extra substrate during hydrolysis appeared to boost the hydrolysis reaction via a “restarting effect,” washed substrate was used. In separate experiments, we have observed a significantly elevated enzymatic hydrolysis of pretreated barley straw with washed substrate compared to unwashed acid-steam-pretreated barley straw (unpublished data); washing or simple water extraction presumably removes acid and any eventual inhibitory substances that inhibit cellulolytic enzymes from Trichoderma sp. [19]. We therefore ascribe the difference between our data and the results of Eriksson et al. [11] with substrate loading to be an effect of differences in substrate washing as washing may remove inhibitory substances in the pretreated lignocellulosic material. Hence, although washing of substrate can result in boosting of the hydrolysis in substrate loading, substrate washing is

![Fig. 1](image-url)

**Fig. 1** Sequential addition of substrate. Glucose yield expressed as gram of glucose per gram of DM in the reaction vs reaction time. The arrows indicate times where additional substrate was loaded into the reactions. Legends refer to the substrate DM loading in percent by weight of the reaction (see “Methods” for codes)
not feasible in industrial reactions. Rather, the results point at the potential importance of developing efficient pretreatment strategies that avoid the use of acid or that avoid carrying the inhibitors into the subsequent reactions.

Viscosity Changes During Hydrolysis

Mild acid pretreatment followed by steam pretreatment of lignocellulosic residues such as straw produce highly viscous heterogeneous substrate slurries containing long entangled fibers, which present a challenge for rheological measurements. Previously, the helical impeller method has been applied to determine the viscosity of pretreated corn stover [20]. Different techniques, including the helical impeller and vane spindle, have been applied for viscosity estimates of fermentation broths of filamentous microorganisms, both with consistent correlation [21, 22]. Vane spindles are recommended for evaluation of flow behavior of various non-Newtonian filamentous fiber suspensions because these spindles leave the substrate structure undisturbed when immersed into the suspension and therefore allow more accurate measurements [22–24].

In all reactions the apparent viscosity decreased with increasing shear rates typically found for non-Newtonian liquids. Furthermore, as expected, the viscosity also decreased with reaction time as hydrolysis progressed (Fig. 2). During the viscosity measurements the flow of the substrate suspensions appeared to gradually change from laminar to turbulent at shear rates >20/s during measurement (Fig. 2). The slight increase in viscosity observed at high shear rates (>20/s) is presumably because of such turbulent flow of the substrate at high shear rates in these reactions and not a result of an actual increase in viscosity. Therefore, only apparent viscosities measured below this shear rate were considered in the subsequent evaluation of the reactions. Because the viscosity response to shear rate was proportional in all reactions at shear rates of less than 20/s, the reactions could be compared at a single

![Fig. 2 Viscosity (mPa/s) versus shear rate (/s) for the reaction loaded with 15% w/w DM](image)
shear rate and for the specific shear rate of 11.45/s at which all measurements at different time points for the different reactions were within the linear range. This shear rate of 11.45/s is marked with a vertical line in Fig. 2 and was used as a benchmark for comparing viscosities during the different hydrolytic reactions (Fig. 3).

With all the different enzyme/substrate reactions, the decrease in viscosity was found to be most pronounced within the first 6 h of reaction (Fig. 3). As expected, samples with lower DM had lower viscosity throughout the reaction. However, beyond 48 h, all the reactions having constant substrate loading were at a similar, low viscosity level of 20–54 mPa/s (Fig. 3). The results also indicated that after 6 h of reacting, the hydrolysis reactions containing 10 and 15% w/w DM began to flow (at shorter reaction times the reactions with these high substrate concentrations were extremely dense, and it was impossible to measure the viscosity at the time of enzyme addition). The effect of enzyme loading was readily seen in the reactions 5+5+5% and the 10+5%, which had a higher enzyme to substrate loading in the beginning of the reaction before additional substrate was loaded (Table 1). As a result of these high enzyme/substrate ratios, the viscosity of each of these reactions decreased quickly during the first few hours of the reactions—much faster than the reactions with constant substrate loading (Fig. 3). When the final portion of additional substrate had been added at 24 h, the subsequent measurement at 48 h showed that the viscosity had increased only slightly in both reactions: 80 and 240 mPa/s for the 5+5+5% and 10+5%, respectively, compared to 90 mPa/s for the 15% DM reaction (Fig. 3). The viscosity of these samples continued to decrease to similar levels of

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**Fig. 3** Summary of viscosity data from all reactions for the specific shear rate 11.45/s vs reaction time. Legends refer to the substrate DM loading in percent by weight of the reaction (see “Methods” for codes). Shown are averages of 20 measurements for each shear rate. The enzyme dosage was 7.5 FPU/g DM of Celluclast and 13 CBU/g DM of NS 188 based on the final DM w/w in the reactions corresponding to 0.3 enzyme to DM (E/S). For reaction 5+5+5% this ratio was 255% at the start of reaction, after additional substrate loadings (arrows) this ratio became 0.6 and 0.3. For the 10+5% the E/S was ~74% at the beginning and ~30% after additional substrate loading.
the comparable reactions with full substrate loading at the beginning of the reaction to values ranging from 20 mPa/s (5% w/w DM) to 85 mPa/s (10%+5%).

Quantifying Viscosity Changes

The power law (Eq. 2) has frequently been used to describe non-Newtonian flow behavior [20, 25, 26]. The power law parameters enable prediction of the viscosity for the total solids loading when changing the shear rate, thus helping to determine the force needed to pump the substrate slurry. The dependence of the consistency index and the power law index to the extent of hydrolysis in the different reactions is shown in Table 4. As the substrate became more hydrolyzed and the lignocellulose substrate lost its structure, the consistency index, $K_{pl}$, decreased for the reactions with constant substrate loading (Table 4). The $K_{pl}$ values of the 5 and 10% w/w DM differed by several orders of magnitude after only 6 h of hydrolysis, but after 72 h the $K_{pl}$ values were relatively similar, ∼5 to 5.8 Pa·s (Table 4).

This similarity indicated that the total degradation of cellulose and hence loss of substrate structure had occurred to practically the same extent in these two reactions. The $K_{pl}$ value of 15% w/w DM reaction at 6 h of reaction was the highest of all, whereas after 72 h the $K_{pl}$ values of the 10%+(5%+E) and the “15%” were similar; 185 and 188 Pa·s, respectively (Table 4). Surprisingly, the corresponding $K_{pl}$ values of the other substrate-fed reactions were higher (Table 4), indicating that the reactions with extra substrate added might not have been as efficiently hydrolyzed as the 15% w/w DM reaction. Furthermore, the rapid hydrolysis as an effect of high enzyme to substrate ratio in the 5+5+5% reaction is readily seen from the equal size of the $K_{pl}$ and power law index after 6 h of hydrolysis compared to the $K_{pl}$ of the 5 and 10% w/w DM reactions after 72 h (Table 4). Generally, the power law indices increased during the course of hydrolysis, reflecting the trend of decreasing viscosity with reaction time as a result of the loss of substrate structure, i.e., presumably a consequence of both the decreasing cellulose degree of polymerization and a structural collapse with increased porosity, as the cellulose matrix was degraded.

Sequential Addition of Substrate and Enzyme

Glucose Concentrations Based on the previous results it appears that enzyme loading is a more important parameter than substrate availability. This conclusion agrees well with the

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**Table 4** Evolution of power law parameters: consistency index ($K_{pl}$) and power law index ($n$) during enzymatic hydrolysis with different types of sequential substrate and substrate plus enzyme loadings.

<table>
<thead>
<tr>
<th>Substrate DM loading (% w/w)</th>
<th>6 h $K_{pl}$</th>
<th>6 h $n$</th>
<th>72 h $K_{pl}$</th>
<th>72 h $n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5+5+5%</td>
<td>5.1</td>
<td>1.54</td>
<td>261.9</td>
<td>0.36</td>
</tr>
<tr>
<td>10+5%</td>
<td>1,229</td>
<td>0.34</td>
<td>526.8</td>
<td>0.27</td>
</tr>
<tr>
<td>5%+(5%+E)+(5%+E)</td>
<td>111.5</td>
<td>0.48</td>
<td>362.9</td>
<td>0.33</td>
</tr>
<tr>
<td>10%+(5%+E)</td>
<td>1,859</td>
<td>0.09</td>
<td>188.4</td>
<td>0.40</td>
</tr>
<tr>
<td>5%</td>
<td>77.1</td>
<td>0.54</td>
<td>4.9</td>
<td>1.56</td>
</tr>
<tr>
<td>10%</td>
<td>2,003</td>
<td>0.05</td>
<td>5.8</td>
<td>1.55</td>
</tr>
<tr>
<td>15%</td>
<td>16,356</td>
<td>0.07</td>
<td>185.4</td>
<td>0.47</td>
</tr>
</tbody>
</table>

Shear rate range 4.68–23.08/s. $K_{pl}$ is given at Pascal second; $n$ is dimensionless. $R^2$ was ≥0.9.

*For codes please see “Methods.”
previously reported finding that addition of extra Cel7A was able to boost the glucose release from steam-pretreated spruce [11]. However, when adding a relatively high enzyme loading from the start of reaction, the odds of having relatively more enzyme non-productively adsorbed to lignin may also increase, and the full effect of the extra enzymes added may not be obtained during the later stages of the reaction. To elucidate whether addition of enzyme simultaneously with the substrate loading would boost the cellulose hydrolysis and keep the initial fast rate of hydrolysis, two experiments where carried out starting at 5 and 10% w/w DM and then extra substrate and more enzymes were added, thus keeping the enzyme/substrate ratio constant during the hydrolysis reaction (Table 1).

When additional substrate was added together with additional enzyme, the glucose concentration in the reaction starting at 5% w/w DM increased when the substrate loading was increased from 5 to 10% w/w DM after 6 h of hydrolysis (Table 2). Moreover, the glucose concentration continued to increase when the final batch of substrate was added after 24 h (Table 2, 5%+[5%+E]+[5%+E]). The glucose concentration also increased in the 10%+(5%+E) reaction when more substrate was added after 24 h and the final glucose concentration of these two reactions were similar to those obtained by sequential addition of substrate alone (final concentration 62–67 g/l) (Table 2). These results suggest that adding extra enzyme and thus keeping the enzyme to substrate ratio constant but in effect increasing the enzyme concentration (Table 1) can “cancel out” the dilution effect of the glucose concentration observed for the reaction added substrate twice without more enzyme being added (Table 2).

**Glucose Yields** In relation to glucose yield, the reaction, which was supplemented with additional substrate and enzymes, reached a glucose level of 0.39 g/g DM (Fig. 4). This yield was similar to that obtained in the reactions, which had a higher enzyme to substrate ratio before extra substrate was added (compare the 72 h data in Figs. 1 and 4).
results therefore suggest that when more substrate is added there is a lag phase during which the glucose yield is low, irrespective if whether more enzyme is being added or not at the same time. This phenomenon may be related to the mixing in of fresh substrate and enzyme because the substrate near the enzymes may be surrounded by high levels of glucose before proper mixing takes place, and the product inhibition of the enzymes might therefore remain in the vicinity of the enzyme for some time until the fresh substrate is well mixed in. It was observed that when fresh substrate was added the viscosity increased markedly but transiently. However, this increase in viscosity was not quantified but it can be speculated to have a negative impact on the desorption of enzyme and distribution of the enzyme and fresh substrate, which might also explain the lag phase observed in relation to glucose yield.

The viscosity of the reactions having substrate added together with fresh enzyme were at similar levels as the 5+5+5% and the 10+5% reactions, where only substrate was added. The viscosity increased transiently when more substrate was added but returned to a low level after the final 72 h of hydrolysis (42 and 85 mPa/s at the specific shear rate 11.54/s) (Fig. 5).

Thus, in relation to viscosity it was found that a sequential increase of the substrate loading to 15% w/w DM the viscosity was kept at a lower level than the comparable reaction with a constant substrate loading of 15% w/w DM. However, no net benefit in relation to glucose concentration or glucose yield was obtained by sequentially increasing the substrate loading either by adding the full enzyme loading at the beginning of the reaction or when substrate was added simultaneously with the enzymes.

Because there is a clear positive relation between the final glucose concentration and the substrate level, and a negative relation between glucose yields and substrate concentration,
a compromise between the two was found to be 12.5% w/w DM after 72 h of reaction (Fig. 6). This substrate loading would result in a glucose concentration of approximately 44 g/l and a yield of 0.92 g glucose/g substrate DM. A fed-batch strategy for SSF worked well in the case of steam-pretreated spruce where the solids loading was gradually increased up to 10% with an enzyme loading of 37.5 FPU/g cellulose [27]. Based on these results, the authors concluded [27] that application of a more efficient and/or heat stable enzyme system would increase the ethanol yield even further, particularly if separate hydrolysis and SSF was combined. Our present study, which was focusing only on the enzymatic hydrolysis step, was, however, carried out using a much lower enzyme loading of 7.5 FPU/g cellulose to reach a more economically realistic enzyme loading, which obviously affects the glucose yields.

Conclusions

In this study, the highest glucose concentration and highest glucose yields from the barley straw lignocellulose substrate were obtained by employing a constant substrate loading. In general, the viscosity decreased rapidly in all reactions during the first few hours of the reactions. However, in the reactions to which additional substrate was added the viscosity decreased more quickly than in those having constant substrate loading because of the relatively higher initial enzyme/substrate ratios. When extra substrate or substrate plus enzymes were added, the viscosities of the slurries increased and the hydrolytic efficiencies decreased transiently, but in all reactions the viscosity decreased to values below 100 mPa/s after the total reaction time of 72 h. The reactions to which additional substrate was added were subject to dilution, thus decreasing the glucose concentration because the substrate stock was 21% w/w DM; thus, a greater amount of water was added simultaneously with the additional substrate. With the enzymes dosage levels employed and a constant total reaction time of 72 h, the extra added substrate was apparently not efficiently hydrolyzed, most likely because the supplemented fresh substrate was exposed to the enzymes for a relatively shorter time period. To optimize the hydrolysis reaction with respect to glucose concentration and

![Graph with open squares: Glucose concentration (g/l) after 72 h of reaction time. Graph with filled squares: glucose yield (g/g DM) as a function of DM after 72 h of reaction time. Graphs intersect at 12.5% w/w DM](image-url)
glucose yield, an optimal substrate loading was found to be 12.5% w/w DM to be loaded at the beginning of the reaction. However, because the viscosity of the reactions with sequentially added substrate remained at low levels relative to the 15% w/w DM reaction, it seems beneficial to increase the substrate loading gradually in relation to the viscosity.

References

Efficiency of New Fungal Cellulase Systems in Boosting Enzymatic Degradation of Barley Straw Lignocellulose

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This study examined the cellulytic effects on steam-pretreated barley straw of cellulose-degrading enzyme systems from the five thermophilic fungi Chaetomium thermophilum, Thielavia terrestris, Thermosascus aurantiacus, Corynebacterium thermophilus, and Myceliophthora thermophila and from the mesophilic Penicillium funiculosum. The catalytic glucose release was compared after treatments with each of the crude enzyme systems when added to a benchmark blend of a commercial cellulase product, Celluclast, derived from Trichoderma reesei and a β-glucosidase, Novozym 188, from Aspergillus niger. The enzymatic treatments were evaluated in an experimental design template comprising a span of pH (3.5–6.5) and temperature (35–65 °C) reaction combinations. The addition to Celluclast + Novozym 188 of low dosages of the crude enzyme systems, corresponding to 10 wt % of the total enzyme protein load, increased the catalytic glucose yields significantly as compared to those obtained with the benchmark Celluclast + Novozyme 188 blend. A comparison of glucose yields obtained on steam-pretreated barley straw and microcrystalline cellulose, Avicel, indicated that the yield improvements were mainly due to the presence of highly active endoglucanase activity/activities in the experimental enzyme preparations. The data demonstrated the feasibility of boosting the widely studied T. reesei cellulase enzyme system with additional enzymatic activity to achieve faster lignocellulosic degradation. We conclude that this supplementation strategy appears feasible as a first step in identifying truly promising fungal enzyme sources for fast development of improved, commercially viable, enzyme preparations for lignocellulose degradation.

Introduction

Significant research efforts have been invested in evaluating and understanding the enzymatic hydrolysis of lignocellulosic substrates by cellulases produced by the fungus Trichoderma reesei (1–5). Commercial products of various T. reesei isolates have long been available for cereal foods, brewing, and fruit and vegetable processing and have also been widely evaluated and applied in relation to bioethanol production processes (4, 5). T. reesei secretes high amounts of enzymes, up to 40 g L−1, that comprise a battery of endoglucanase, cellobiohydrolase, β-glucosidase, and different hemicellulolytic activities that catalyze the degradation of the cellulose and hemicellulose of plant cell walls (6–7).

Prior to enzymatic hydrolysis most lignocellulosic substrates undergo some sort of pretreatment to increase the accessibility of the substrate to enzymatic attack. The pretreatment should preferably result in removal of lignin and increase the available surface area and the substrate porosity and may also solubilize the hemicellulose (8, 9). In particular, the removal of lignin has a large influence on the rate and extent of enzymatic hydrolysis of the substrate (10).

However, despite pretreatment of the lignocellulosic substrates, the respective activities in T. reesei cellulase products appear not always to be present in optimal ratios for degradation of lignocellulose (11, 12). One particular activity that is often limiting is β-glucosidase. In T. reesei this activity is partly mycelia-bound and hence not efficiently recovered during the industrial enzyme production (13–15). The low β-glucosidase activity results in incomplete hydrolysis of cellulbiose, which in turn results in product inhibition of the T. reesei enzyme systems (16, 17). Hence, supplementation with extra β-glucosidase is usually required, and this supplementation is often accomplished by addition of the Novozym 188 β-glucosidase preparation derived from Aspergillus niger (2, 11, 17). In addition, relatively large quantities of the commercial T. reesei cellulase products, including for example the Celluclast + Novozym 188 mixture, are needed for complete conversion of cellulose. This is presumably because of the low accessibility to interior bonds in insoluble substrates resulting in low rates of interior β-linked bonds cleavage (7). Nevertheless, both of these requirements contribute to making current commercially available T. reesei enzyme products unsuitable for large-scale production of bioethanol (18, 19). Because of the high levels of cellulases produced by T. reesei strains, these seem to be a well-suited starting point for obtaining improved cellulase hydrolysis via boosting of certain cellulase activities, rather than by complete replacement with a novel system from another cellulytic fungus. Crude enzyme extracts of various strains of Penicillium have been shown to exert high activity on lignocellulose compared to Cellulase (12) and high filter paper activity compared to culture broth from the T. reesei Rut C30 strain (20). These data corroborate the potential of finding new,
more specific cellulases within the genus *Penicillium* to supplement to the traditional cellulase products.

Because enzyme activity is expected to increase and often almost doubles for every 10 °C increase in temperature (up to a certain maximum temperature where inactivation occurs) (21), a way to reduce the production costs of ethanol from lignocellulose is to carry out the enzymatic hydrolysis at high temperature. This could potentially reduce the reaction time, the retention time in the reactor, and the required enzyme loading. During short-term lignocellulose hydrolysis Celluclast is stable at 50 °C (2), and short-term stability assays have shown that maximal activity on pure cellulose substrates is retained even up to 65 °C (22). Some filamentous fungi produce cellulases that retain relatively high cellulase-degrading activity at temperatures of 50–70 °C, particularly species such as *Thielavia terrestris*, *Thermoa sp. aurantiius* , *Chaetomium thermophilum*, *MyCeliothora thermophila*, and *Corynascus thermophilus* (23–26). Other fungi, for example from the *Penicillium* family such as *P. funiculosum*, display a broad profile of cellulytic enzymes, particularly with high *β*-glucosidase activity compared to that of some *T. reesei* strains (27). We speculated that enzymes from these fungal strains might enhance the cellulytic effect of Celluclast if the two enzyme systems were mixed.

This study was therefore undertaken to assess the hypothesis that boosting of a widely used *T. reesei* cellulase product by addition of enzymes from other fungi might be a fruitful avenue for obtaining improved cellulose hydrolysis. This paper thus presents an evaluation of the applicability of selected fermentation broths from various thermophilic filamentous fungi to supplement the traditional cellulase products.

**Methods**

Substrate. A solid cellulose-rich fraction from steam-pretreated barley straw was provided by Professor Guido Zacchi at the Department of Chemical Engineering, Lund University, Sweden and used as substrate in all experiments. In brief, the straw had first been subjected to soaking in 1% w/v sulfuric acid and afterward to steam pretreatment at 170 °C for 5 min. Immediately after treatment, the substrate was frozen and transported to Denmark. To determine the substrate composition the standard procedure from the U.S. National Renewable Energy Laboratory (NREL) was used. The cellulose content was calculated to comprise 40 ± 2% of the total solids (TS) based on the amount of glucose released. The TS was determined based on the substrate weight loss after 18 h drying at 105 °C. Arabinose and xylose were calculated to comprise 2 ± 0.2% and 8 ± 1% of the total solids, respectively. The residual solids were not accounted for but were supposed to consist of lignin and other acid-insoluble solids. As a model substrate, Avicel from Merck Biosciences KGaA, (Darmstadt, GE) was used.

**Enzymes and Protein Methods.** Celluclast derived from *T. reesei* was from Novozymes A/S (Bagsvaerd, Denmark). The Cellulast preparation had a declared activity of 709 EGU- g−1 (EGU = endoglucanase units) and the filter paper unit (FPU) activity was 67 ± 1.4 FPU- g−1 using the NREL standardized filter paper assay (NREL Laboratory analytical procedure 002 (1996)) was used. The cellulose content was calculated to comprise 40 ± 2% of the total solids (TS) based on the amount of glucose released. The TS was determined based on the substrate weight loss after 18 h drying at 105 °C. Arabinose and xylose were calculated to comprise 2 ± 0.2% and 8 ± 1% of the total solids, respectively. The residual solids were not accounted for but were supposed to consist of lignin and other acid-insoluble solids. As a model substrate, Avicel from Merck Biosciences KGaA, (Darmstadt, GE) was used.

**Table 1. List of Fungal Sources Evaluated in This Paper, Their Protein Content and FPU Activity**

<table>
<thead>
<tr>
<th>Source</th>
<th>Protein Conc (g/L)</th>
<th>Activity (FPU mg−1 protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chaetomium thermophilum (C.t)</td>
<td>1</td>
<td>0.03</td>
</tr>
<tr>
<td><em>Thiolesa terrestris</em> (T.t)</td>
<td>2.1</td>
<td>0.13</td>
</tr>
<tr>
<td><em>Thermoascus aurantiius</em> (T.a)</td>
<td>2.1</td>
<td>0.18</td>
</tr>
<tr>
<td><em>Corynascus thermophilus</em> (C.t)</td>
<td>1.5</td>
<td>0.05</td>
</tr>
<tr>
<td><em>MyCeliothora thermophila</em> (M.t)</td>
<td>0.9</td>
<td>0.06</td>
</tr>
<tr>
<td><em>Penicillium funiculosum</em> (P.f)</td>
<td>1.4</td>
<td>0.03</td>
</tr>
</tbody>
</table>

**Table 2. Reaction Conditions, Temperature and pH, and Number of Samples Encompassed by the Surface-Response Design Applied in the Present Study; Center Point Reactions (pH 5, 50 °C) Were Repeated Three Times**

<table>
<thead>
<tr>
<th>pH</th>
<th>Temp (°C)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>40</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>40</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>3</td>
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<td>3</td>
</tr>
<tr>
<td>5</td>
<td>65</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>3</td>
</tr>
</tbody>
</table>

**Enzymatic Hydrolyses.** Each of the enzyme preparations (fungal broths) was evaluated in the same statistically designed, experimental template. This experimental template was a randomized, quadratical central composite circumscribed (CCC) response surface design with the factors reaction temperature 35–65 °C [40–60 °C] and pH 3.5–6.5 [4–6] as experimental variables and with a reaction time of 6 h and a substrate concentration of 2% TS by weight held constant. This resulted in nine different process combinations with two star points for each variable and three replicated center points (Table 2). Template experiments were done twice for evaluation of each broth sample and thrice for the benchmark and the double benchmark enzyme loadings. The factor ranges in the experimental plan were chosen to reflect relevant industrial processing requirements. Prior to enzymatic hydrolysis, the pretreated barley straw was resuspended in 0.05 M NaOAc/HCl, pH 5. The hydrolyses were carried out directly on this substrate in 2 mL Eppendorf tubes incubated in thermostated thermomixers (Comfort by Eppendorf AG, Hamburg, GE) with shaking at 1000 rpm. After incubation the reactions were terminated by boiling for 10 min. Each sample was then centrifuged, and the supernatant was collected, filtered through a 0.2 μm Millex-GN nylon filter (Millipore, Glostrup, DK), and subjected to
Hydrolysis was carried out at pH 5, 50 °C supplemented with 1 mg of NS 188 (∼13 CBU per gram TS). Both reactions were depicted as closed and open symbols, respectively. Both reactions were supplemented with 1 mg of NS 188 (∼13 CBU per gram TS). The hydrolysis was carried out at pH 5, 50 °C with 2 wt % of TS.

HPAEC analysis, see below, for glucose and cellobiose quantification or subjected to colorimetric assessment of reducing ends.

High Performance Anion Exchange Chromatography (HPAEC). Glucose and cellobiose (xylose, arabinose, and galactose) were separated on a Dionex BioLC system equipped with a CarboPac PA1 column (4 × 250 mm) (Dionex Denmark A/S, Hvidovre, DK) and a CarboPac PA1 guard column (4 × 50 mm). Samples were eluted isocratically with 0.02 M KOH at a flow rate of 1 mL·min⁻¹; and analytes were detected by electrochemical detection in a pulsed amperiometric detection mode.

Quantification of Reducing Ends. The DNS reagent prepared as for determination of FPU was added in equal volume to each supernatant from the hydrolysis reactions, and the resulting mixtures were incubated at 100 °C for 10 min. After cooling for 20 min, the samples were diluted with ddH₂O, and the absorbances were measured at 550 nm. A dilution series of glucose was used to determine the amount of reducing sugars in the samples.

Statistical Analysis. SAS Jump version 5.1 (SAS Institute Inc, Cary, NC) was used to create the surface response template and to aid the multivariate data analysis. Statistical significance was established at P ≤ 0.05. Differences in the yields of other experiments were determined by one-way analysis of variance, where the 95% confidence intervals were calculated from pooled standard deviations (Minitab Statistical Software, Addison-Wesley, Reading, MA).

Results and Discussion

Determination of Benchmark Conditions. To define the benchmark conditions for the enzymatic hydrolysis with regard to reaction time, Celluclast + NS 188 loading, and substrate concentration, a comparison of the glucose and cellobiose release and the cellulose conversion was made during prolonged Celluclast + NS 188 treatment of steam-pretreated barley straw substrate at pH 5, 50 °C. Treatment with Celluclast and NS 188 at a dosage of 16 FPU Celluclast and 13 CBU NS 188 per gram TS, equivalent to dosages of 4.6 mg and 1 mg of enzyme protein per gram TS, respectively, gave high rates of glucose release with a yield of 8.8 g·L⁻¹ within the first 6 h of treatment, corresponding to almost 95% cellulose conversion (Figure 1). After the initial 6 h of reaction, the cellulose conversion and thus the glucose release leveled off and increased only marginally during the following 42 h of reaction. After a total reaction time of 48 h the glucose concentration was approximately 9.5 g·L⁻¹ (Figure 1). This final glucose level corresponded to approximately 100% conversion (Figure 1). The cellobiose concentration initially increased transiently but decreased from approximately 0.32 to 0.08 mg·L⁻¹ within the first 6 h (Figure 1).

Effect of substrate loading (total solids percent of reaction volume by weight) on hydrolysis measured as glucose release (□) and calculated as cellulose conversion percentage (C) using a Celluclast dosage per gram reaction of 4.6 mg (∼16 FPU per gram TS) and 2.3 mg (∼8 FPU per gram TS), depicted as closed and open symbols, respectively. Both reactions were supplemented with 1 mg of NS 188 (∼13 CBU per gram TS). The hydrolysis was carried out at pH 5, 50 °C.

Figure 1. Time course of glucose (square symbols) and cellobiose (triangular symbols) release during enzymatic hydrolysis of steam-pretreated barley straw substrate at pH 5, 50 °C using a Celluclast dosage per gram reaction of 4.6 mg (∼16 FPU per gram TS) and 2.3 mg (∼8 FPU per gram TS), depicted as closed and open symbols, respectively. Both reactions were supplemented with 1 mg of NS 188 (∼13 CBU per gram TS). The hydrolysis was carried out at pH 5, 50 °C with 2 wt % of TS.

Figure 2. Effect of substrate loading (total solids percent of reaction volume by weight) on hydrolysis measured as glucose release (□) and calculated as cellulose conversion percentage (C) using a Celluclast dosage of 2.3 mg (∼8 FPU) per gram TS supplemented with 1 mg NS 188 (∼13 CBU) per gram TS. The hydrolysis was carried out at pH 5, 50 °C.

To define the benchmark conditions for the enzymatic hydrolysis with regard to reaction time, Celluclast + NS 188 loading, and substrate concentration, a comparison of the glucose and cellobiose release and the cellulose conversion was made during prolonged Celluclast + NS 188 treatment of steam-pretreated barley straw substrate at pH 5, 50 °C. Treatment with Celluclast and NS 188 at a dosage of 16 FPU Celluclast and 13 CBU NS 188 per gram TS, equivalent to dosages of 4.6 mg and 1 mg of enzyme protein per gram TS, respectively, gave high rates of glucose release with a yield of 8.8 g·L⁻¹ within the first 6 h of treatment, corresponding to almost 95% cellulose conversion (Figure 1). After the initial 6 h of reaction, the cellulose conversion and thus the glucose release leveled off and increased only marginally during the following 42 h of reaction. After a total reaction time of 48 h the glucose concentration was approximately 9.5 g·L⁻¹ (Figure 1). This final glucose level corresponded to approximately 100% conversion (Figure 1). The cellobiose concentration initially increased transiently but decreased from approximately 0.32 to 0.08 mg·L⁻¹ within the first 6 h (Figure 1). Principally the same pattern was found during hydrolysis using half of the Celluclast dosage, i.e., at enzyme loadings of Celluclast and NS 188 of 8 FPU and 13 CBU per gram TS, respectively. However, after 6 h, the cellulose conversion, was only ∼75%, equivalent to release of 7.2 g·L⁻¹ glucose (Figure 1). To allow sufficient room for assessing any potential boosting effects of experimental enzyme extracts added to Celluclast, the lower addition of Celluclast was chosen as the benchmark dose in the boosting experiments. Because of the initial fast reaction rate and in keeping with desired process conditions for separate hydrolysis and fermentation (SHF), the glucose yields were compared after a reaction time of 6 h. The highest glucose concentrations occurred with substrate concentrations beyond 1.75% w/w TS, but the glucose yields were not significantly different between substrate concentrations from 1.75% to 3.5% w/w TS (Figure 2). Since a total solids loading of 2% w/w resulted in approximately 80% cellulose conversion within the 6 h reaction time (Figure 2), this substrate loading was chosen as a base for the evaluation of the fungal fermentation broths.

Evaluation of Fungal Fermentation Broths. To assess the potential cellulytic boosting effect of the novel fungal cellulase systems, a low dosage, corresponding to 10 wt % of the total enzyme protein load, of each of the fungal fermentation broths was supplemented to an enzyme mixture comprising Celluclast and NS 188 (8 FPU and 13 CBU per gram TS, respectively). To evaluate the effect of the pH and temperature on the enzymatic reaction, the hydrolytic efficacy of each of the fungal broths was examined in a surface response experimental template comprising a pH range from 3.5 to 6.5 and a temperature range from 35 to 65 °C (Table 2). There were no statistically significant main or interactive effects of the reaction parameters on any of the fungal broths (data not shown). The cellulose conversion was, however, significantly increased by addition of the fermentation broth samples compared to the yields obtained with the benchmark Celluclast + NS 188 dosage (BM) (Figure 3). Notably, at pH 5 and 50 °C five of the six broth samples, i.e., all except the C. thermophilum sample, gave
glucose yields (and cellulose conversion) that were equivalent to or higher than those obtained with twice the benchmark loading of Celluclast supplemented with NS 188 (Figure 3, black stars).

A few isolated sets of results, e.g., the low glucose yields (cellulose conversion) obtained for the treatments at pH 6, 40 °C with BM + M. thermophila and BM + C. thermophilum and with the BM + C. thermophilum after the treatments at pH 5, 35 °C; pH 4, 40 °C, and pH 6.5, 50 °C (Figure 3, white stars), indicated that the C. thermophilum broth was apparently the least efficient and the most sensitive to pH changes among the broth preparations.

However, within the experimental conditions evaluated, none of the other fungal broths stood out as performing significantly better or worse than the others (Figure 3). P. funiculosum, which is categorized as a mesophile organism, has previously been described to secrete high amounts of cellulases and β-glucosidase (27). In our study the P. funiculosum enzymes also seemed to display a pronounced cellulase boosting effect at 50 °C. In contrast, C. thermophilum, known to produce thermostable cellulases (23), did not show any significant boosting effect on glucose release when added together with Celluclast and NS 188 (Figure 3). The results indicate that the C. thermophilum enzyme broth contained a profile of cellulases similar to those of T. reesei, whereas the broths of the other fungi, which were able to act synergistically with the Celluclast + NS 188 mixture, most likely contained a profile of cellulases different from that of T. reesei. However, further examination of the enzyme profiles of these novel fungal cellulase producers is required to validate this assumption.

The particularly high yields obtained for almost all enzyme mixtures at pH 5, 50 °C could be attributed to the fact that the fungal strains were initially selected because of their high activity specifically at 50 °C. However, the high yields may also reflect the Celluclast optimum rather than the optimum of the fermentation broths since the latter only contributed 10% of the total protein. Nevertheless, the finding that a supplementation dose of the broths equivalent to 10% of the total enzyme protein gave yields that matched the yield obtained with twice the benchmark Celluclast loading (with 13 CBU NS 188) indicated the significant cellulytic boosting potential of the five best broths.

Lowering of the Benchmark Dosage and Production of Reducing Ends. Treatment with the benchmark dosage of Celluclast and NS 188, to which the fermentation broths were added, gave a relatively high cellulose conversion alone, i.e., ~75% conversion within the 6 h reaction (Figures 1 and 3). To ensure that the increase in cellulose conversion brought about by the enzymes in the fermentation broths was not underestimated due to a high Celluclast dosage, the broth samples were also added to half the benchmark dosage of Celluclast supplemented with NS 188. Furthermore, an assessment of reducing ends was done in addition to glucose yield analysis to avoid overlooking any potential differences in the cellulose depolymerization efficiency among endoglucanases in the fermentation broths.

At pH 5, 50 °C corresponding to the center points of the surface response design, the same most efficient five broths, i.e., T. aurantiacus, C. thermophilus, T. terrestris, M. thermophila, and P. funiculosum, resulted in an increase in cellulose conversion when added to a lower dosage of Celluclast (Figure 4). The yields obtained were comparable to those observed in the surface response design (compare Figure 4 with Figure 3 (pH 5; 50 °C)). In addition, the fermentation broth of C. thermophilum, which did not increase the cellulose conversion in the surface response design significantly, was able to increase the cellulose conversion when added at 30 wt % of that of Celluclast protein.

Hence, the determination of reducing ends present in the samples did not indicate any differences in the catalytic liberation of larger oligomers by endoglucanases in the fermentation broths. Rather, the data indicated that the reducing ends produced upon addition of fermentation broths correlated well with the glucose release as determined by HPAEC (Figure 4).

Activity on Pure Cellulose. The fermentation broths were also applied to a pure, more crystalline cellulose substrate, Avicel, and examined under different reaction conditions using the surface response design. This was done in order to assess whether the cellulytic activities of the enzymes present in the
fermentation broths were related to the nature of the substrate. To supplement the glucose quantification, the amount of reducing ends was also quantified. If oligomers larger than cellobiose would be produced upon addition of the fermentation broths, it was expected that the levels of reducing ends would be higher than the neat glucose levels analyzed by HPAEC. The results showed no main effects nor significant interacting effects of pH or temperature with addition of either fermentation broths added must be a result of the production of soluble glucans (although these were not identified), these results suggest the presence of more active endoglucanases or a skewed ratio of endoglucanase and cellobiohydrolases in the crude fermentation broths compared to the well described cellulase spectrum present in Celluclast. These results therefore emphasize that the nature of the substrate has a significant impact on the observed effects of cellulytic enzyme mixtures.

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Previously, differences in the characteristics of microcrystalline cellulose and lignocellulose have been related to differences in synergy effects between T. reesei EGII and CBHI, which on Avicel show lower synergy than on steam-pretreated willow at low conversion rates (29, 30). In line with the arguments for selecting the fungal strains evaluated in this study, it has previously been shown (31) that although culture filtrates of the strain Sporotrichum thermophilum had exo- and endoglucanase activities 10-fold lower than those of T. reesei (FPU activity of 0.12 FPU·mL⁻¹ with a secreted protein content of 0.18 mg·mL⁻¹), S. thermophilum was able to degrade and grow on cellulose paper much faster compared to T. reesei (five times the growth rate of T. reesei). Differences in sugar uptake rate and growth rate may thus play a critical role in fungal growth on cellulose, and for this reason such comparisons of fungal growth rate data do not allow a firm conclusion to be drawn regarding the catalytic efficiency of the cellulases secreted.

Rather, the performance of different cellulase systems should be evaluated separately from the fungal producer’s growth and in close relation to the process scheme, e.g., in high-temperature saccharification and on the relevant substrates as has also been the main observation in the current study and others (31).
Conclusions

The results showed that it is possible to increase the cellulytic activity of Celluclast supplemented with β-glucosidase (NS 188) by addition of crude fermentation broths from various fungal sources. Although the fungal sources were classified as thermophilic, there were no effects or shifts in the temperature optimum with the fermentation broths. Applied compared to that asserted by Celluclast + NS 188 alone. Since addition of the fermentation broth did not result in an increase in glucose production when applied to Avicel but did increase the amount of reducing ends produced, it is concluded that the experimental fungal broths contained endoglucanase activity(ies) that were more active on pure cellulose than those present in the T. reesei Celluclast preparation. Further studies are required to assess whether these high endoglucanase activities were due to the production of particular enzyme activity or a result of a particularly propitious profile of endoglucanase, cellobiohydrolase, and β-glucosidase activities.

Acknowledgment

This research was partially financed by the European Commission Framework V, contract no. NNE5/2001/685 (The Babilafuente Bioethanol Project).

References and Notes

(1) Szengyel, Z.; Zacchi, G.; Varga, A.; Réczey, K. Cellulase, and particularly propitious profile of endoglucanase, cellobiohydrolase, and β-glucosidase activities.

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References and Notes


Accepted for publication February 22, 2006.

BPH050361O