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

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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,

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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 cellobiohydrolase 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 1 Enzyme dosage scheme for the different reactions as calculated as enzyme/substrate concentration (E/S %), and as E concentration in the total slurry (E conc. %), respectively.

Substrate addition mode	E/S % ((g enzyme/g DM)×100)			E conc. % ((g enzyme/g liquids)×100)		
	0–6 h	6–24 h	24–72 h	0–6 h	6–24 h	24–72 h
5+5+5%	255	85.3	42.7	13.4	9.65	6.75
10+5%	73.8	73.8	29.6	8.20	8.20	5.05
5%+(5%+E)+(5%+E)	25.7	25.7	25.7	1.35	2.90	4.05
10%+(5%+E)	25.7	25.7	25.7	2.85	2.85	4.40
5%	25.7	25.7	25.7	1.35	1.35	1.35
10%	25.7	25.7	25.7	2.85	2.85	2.85
15%	25.7	25.7	25.7	4.55	4.55	4.55

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, γ, was calculated by the following equation:

$$\gamma = \frac{2 \cdot \omega \cdot R_c^2 \cdot R_b^2}{r^2 \cdot \left(R_c^2 - R_b^2\right)} \tag{1}$$

where ω 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.

$$\tau = K_{pI}\,\gamma^n \Rightarrow \eta = K_{pI}\,\gamma^{n-1} \tag{2}$$

where τ is the shear stress, γ the shear rate, η the viscosity, K_{pI} the consistency index, and n the power law index.

Table 2 Substrate composition calculated as percent by weight of DM.

Substrate composition[a]	Substrate addition mode
Arabinose	1.8±0.1%
Xylan[a]	13±2%
Galactose	0.4±0.01%
Glucan[a]	58±2%
Lignin (Klason)	30±0.8%

[a] Calculated as if bound in polymer based on the monosaccharide concentration as determined by high-performance liquid chromatography

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 than in the comparable 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

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

Substrate DM loading (% w/w)[a]	6 h	24 h	72 h
5+5+5%	25±3.6[d]	43±1.1[c]	63±4.5[b]
10+5%	31±0.7[d]	44±7.2[c]	66±1.8[b]
5%+(5%+E)+(5%+E)	15±0.7[e]	49±5.3[c]	62±7.6[b]
10%+(5%+E)	24±1.7[d]	44±7.2[c]	67±4.0[b]
5%	13±1.4[e]	24±2.7[d]	28±1.1[d]
10%	22±0.6[d]	41±0.9[c]	47±3.5[c]
15%	31±1.4[d]	60±1.0[b]	78±5.7[a]

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

[a] For codes, please see "Methods."

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%

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

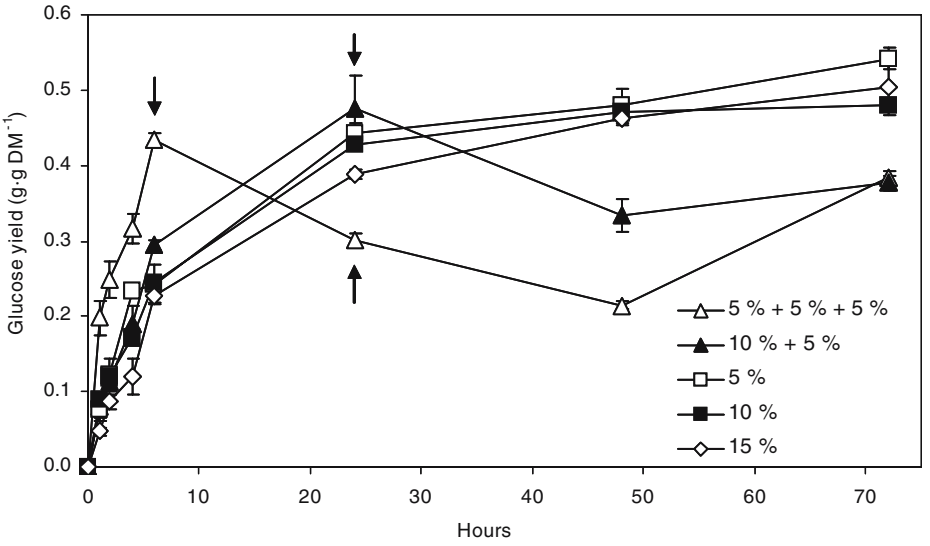

Fig. 1 Sequential addition of substrate. Glucose yield expressed as gram of glucose per gram of DM in the reaction vs reaction time. The arrows indicate times where additional substrate was loaded into the reactions. Legends refer to the substrate DM loading in percent by weight of the reaction (see "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

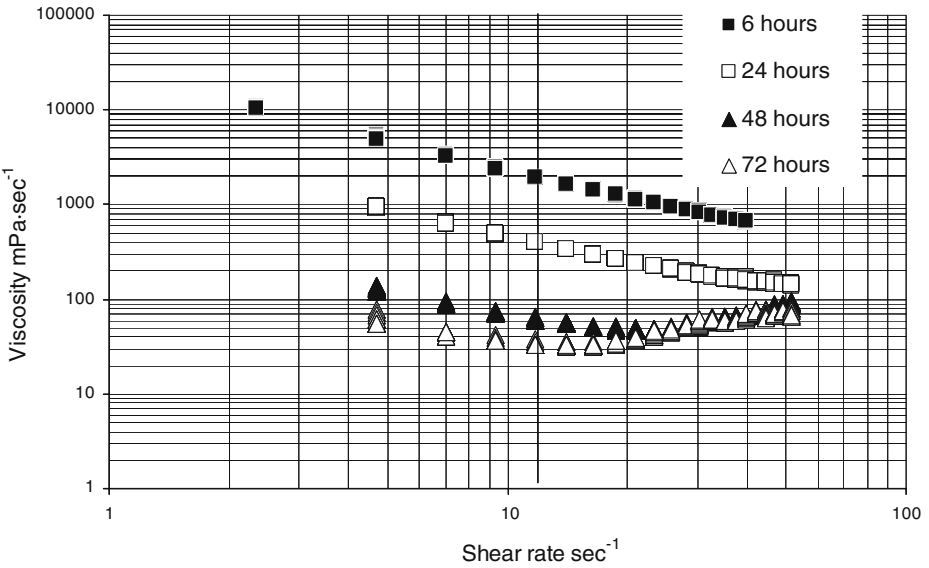

Fig. 2 Viscosity (mPa/s) versus shear rate (/s) for the reaction loaded with 15% w/w DM

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

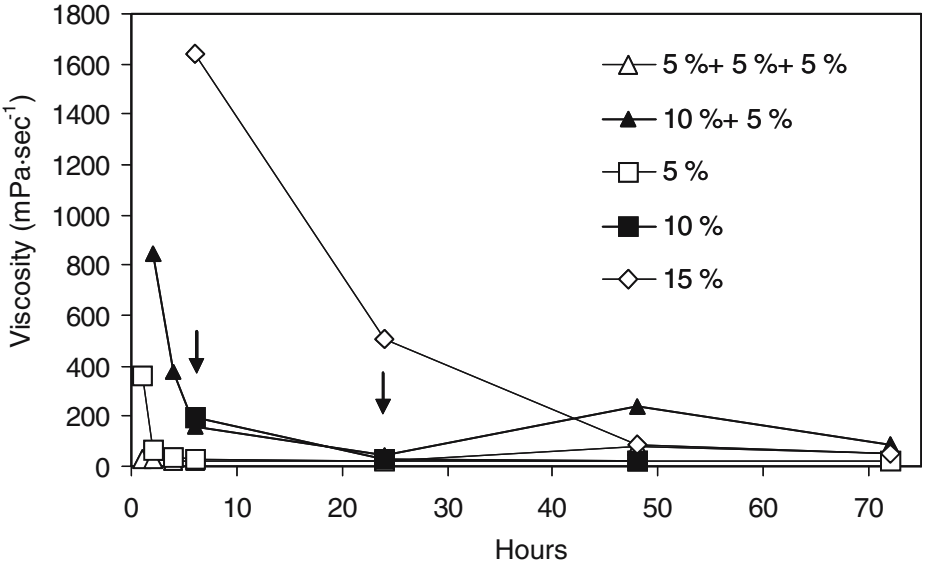

Fig. 3 Summary of viscosity data from all reactions for the specific shear rate 11.45/s vs reaction time. Legends refer to the substrate DM loading in percent by weight of the reaction (see "Methods" for codes). Shown are averages of 20 measurements for each shear rate. The enzyme dosage was 7.5 FPU/g 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

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

Substrate DM loading (% w/w)[a]	6 h		72 h	
	K_{pI}	n	K_{pI}	n
5+5+5%	5.1	1.54	261.9	0.36
10+5%	1,229	0.34	526.8	0.27
5%+(5%+E)+(5%+E)	111.5	0.48	362.9	0.33
10%+(5%+E)	1,859	0.09	188.4	0.40
5%	77.1	0.54	4.9	1.56
10%	2,003	0.05	5.8	1.55
15%	16,356	0.07	185.4	0.47

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

[a] For codes please see "Methods."

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_{pI}, decreased for the reactions with constant substrate loading (Table 4). The K_{pI} 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_{pI} 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_{pI} value of 15% w/w DM reaction at 6 h of reaction was the highest of all, whereas after 72 h the K_{pI} values of the 10%+(5%+E) and the "15%" were similar; 185 and 188 Pa·s, respectively (Table 4). Surprisingly, the corresponding K_{pI} 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_{pI} and power law index after 6 h of hydrolysis compared to the K_{pI} 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

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). These

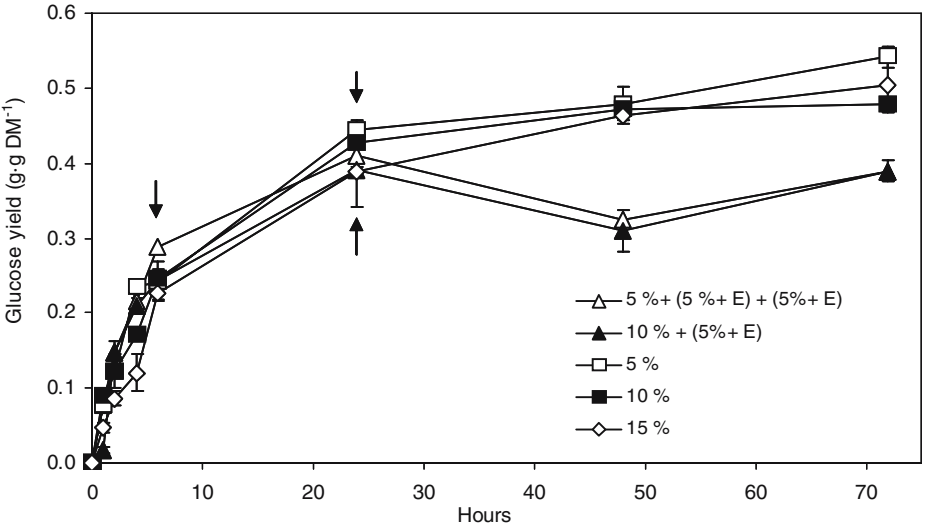

Fig. 4 Sequential addition of substrate and enzyme. Glucose yield expressed as gram of glucose per gram of DM in the reaction vs reaction time. The arrows indicate times where additional substrate was loaded into the reactions. Legends refer to the substrate DM loading in percent by weight of the reaction (see "Methods" for codes)

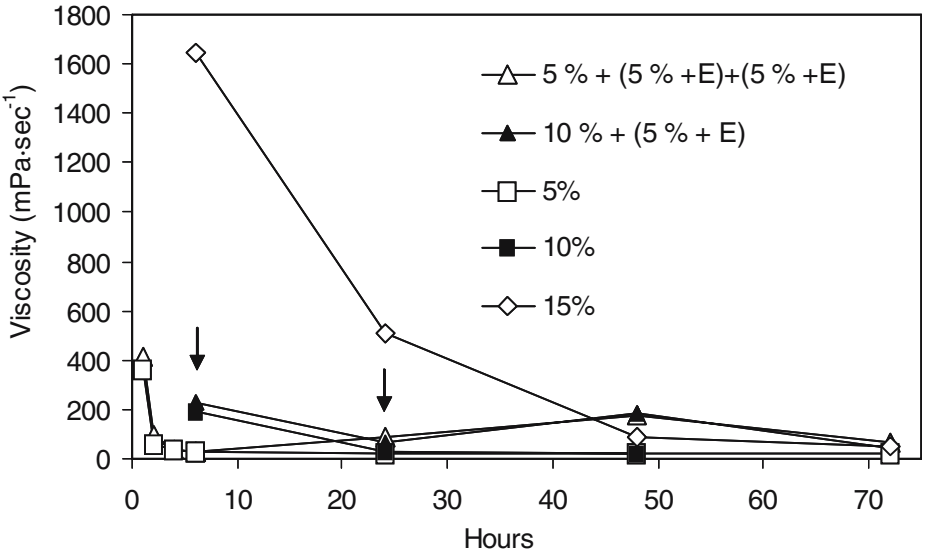

Fig. 5 Summary of viscosity data from all reactions for the specific shear rate of 11.45/s vs reaction time. Legends refer to the substrate DM loading in percent by weight of the reaction (see "Methods" for codes). Shown are averages of 20 measurements for each shear rate. The enzyme dosage was 7.5 FPU/g DM of Celluclast and 13 CBU/g DM of NS 188 based on the final DM w/w of reaction and kept a constant enzyme to substrate level of 25.7 at all time; thus the reaction, which was added with extra substrate, was also added with Celluclast and NS 188 accordingly

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,

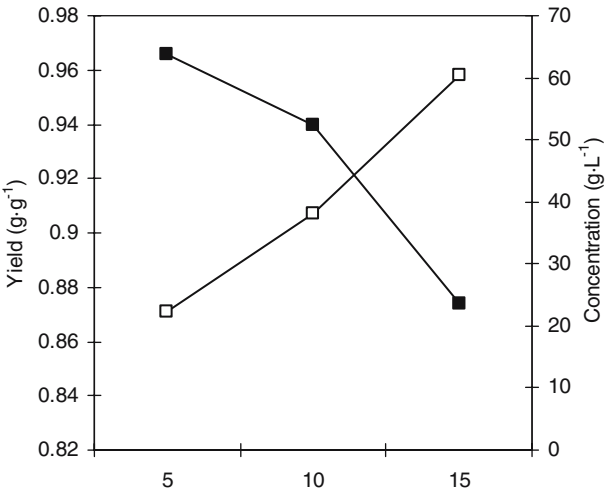

Fig. 6 Graph with open squares: Glucose concentration (g/l) after 72 h of reaction time. Graph with filled squares: glucose yield (g/g DM) as a function of DM after 72 h of reaction time. Graphs intersect at 12.5% w/w DM

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

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.

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