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Steam Explosion Pretreatment of Beechwood. Part 2: Quantification of Cellulase Inhibitors and Their Effect on Avicel Hydrolysis

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Abstract: Biomass pretreatment is a mandatory step for the biochemical conversion of lignocellulose to chemicals. During pretreatment, soluble compounds are released into the prehydrolyzate that inhibit the enzymatic hydrolysis step. In this work, we investigated how the reaction conditions in steam explosion pretreatment of beechwood (severity: 3.0–5.25; temperature: 160–230 °C) influence the resulting amounts of different inhibitors. Furthermore, we quantified the extent of enzyme inhibition during enzymatic hydrolysis of Avicel in the presence of the prehydrolyzates. The amounts of phenolics, HMF, acetic acid and formic acid increased with increasing pretreatment severities and maximal quantities of 21.6, 8.3, 43.7 and 10.9 mg/$g_{beechwood}$, respectively, were measured at the highest severity. In contrast, the furfural concentration peaked at a temperature of 200 °C and a severity of 4.75. The presence of the prehydrolyzates in enzymatic hydrolysis of Avicel lowered the glucose yields by 5–26%. Mainly, the amount of phenolics and xylose and xylooligomers contributed to the reduced yield. As the maximal amounts of these two inhibitors can be found at different conditions, a wide range of pretreatment severities led to severely inhibiting prehydrolyzates. This study may provide guidelines when choosing optimal pretreatment conditions for whole slurry enzymatic hydrolysis.

Keywords: steam explosion pretreatment; enzymatic hydrolysis; inhibition; HMF; phenolics; furfural

1. Introduction

The enhanced utilization of lignocellulosic biomass as an alternative feedstock to petroleum for the production of energy carriers and chemicals is a commonly accepted means to mitigate climate change [1,2]. Biorefineries based on the sugar platform convert lignocellulosic biomass to fuels and chemicals via a process sequence consisting of the principal steps of feedstock pretreatment, enzymatic hydrolysis and fermentation [3]. The pretreatment step is mandatory to achieve high sugar yields in the enzymatic hydrolysis step for most types of biomass [4–6]; however, some exemptions such as corn pericarp are known [7]. However, it is also well known that during pretreatment soluble compounds are generated that inhibit the enzymatic hydrolysis and/or the fermentation step [8]. These compounds include soluble monomeric or oligomeric sugars, lignin derived phenolic compounds, sugar degradation products such as furfural and hydroxymethylfurfural (HMF) and organic acids such as acetic and formic acid [8–12]. An integrated and economically feasible bioconversion process should be able to deal with such inhibitors and should not require additional washing and detoxification steps [13,14]. Thus, it is important to understand how these inhibitors affect enzymatic hydrolysis and

fermentation in the "whole slurry" mode, where the liquid and the solid phase after pretreatment are processed together [14,15].

Previous work has shown that hydrolysis yields in the presence of various prehydrolyzates (the liquid phase after biomass pretreatment) are decreased by up to 70% (see also Supplementary Materials, Table S1). For instance, Garcia-Aparicio et al. showed that the prehydrolyzate of steam-exploded barley straw reduced the enzymatic hydrolysis yield by 25% [16]. Kim et al. found that the prehydrolyzate from liquid hot water (LHW) pretreated maple reduced the 72 h hydrolysis yield of Solka Floc by 57% [17], while the prehydrolyzate from LHW pretreated sugarcane bagasse reduced the glucose yield by 20% and 45% for pretreatment temperatures of 180 and 200 °C, respectively [18]. The extent of cellulase inhibition depends on the type of biomass and the chosen pretreatment methods. The phenolic compounds generated during SO_2 catalyzed steam explosion of lodgepole pine exhibited a significantly higher inhibition than the equal concentration of phenolics from similarly pretreated poplar [19]. Phenolics derived from LHW pretreatment of corn stover have been shown to be less inhibitory than phenolics isolated from ammonia fiber expansion pretreatment but more inhibitory than those from dilute acid and alkaline pretreatment [20].

The inhibition by the prehydrolyzate can partly be overcome by increasing the enzyme dosage: to reach 75% cellulose conversion in whole slurry enzymatic hydrolysis of acid pretreated corn stover, about 1.5–2.7 times more enzymes are needed than the amount necessary to reach 85% cellulose conversion in washed solids enzymatic hydrolysis [21]. In addition, not all enzymes react identically to inhibitors. For instance, Zhai et al. demonstrated that the newest generation of Novozymes cellulase cocktails (CTec3) is less prone to inhibition than the former enzyme mixture Celluclast [22]. More recent work showed that beta-glucosidase and cellobiohydrolases (CBH) were mainly inhibited by sugars present in the prehydrolyzates, while xylanases and CBH are deactivated by the phenolics [15]. Cellulases produced by *Chrysoporthe cubensis* and *Penicillium pinophilum* tolerated an up to approximately 100 times higher phenolics concentration than cellulolytic enzymes of *Aspergillus niger* and *Trichoderma reesei* [23].

Taken together, the generally detrimental effect of prehydrolyzates derived from different pretreatments of different biomasses on enzymatic hydrolysis of lignocellulosic biomass has been clearly demonstrated in former work. However, it has rarely systematically been investigated how the pretreatment conditions (i.e., temperature and pretreatment time) influence the composition of the resulting mix of inhibitors, if the same feedstock and the same pretreatment method is applied. For example, Djioleu et al. characterized 24 different prehydrolyzates of dilute acid pretreated switchgrass, but, to the best of our knowledge, no such comprehensive dataset exists for autohydrolysis pretreatments. Thus, we analyzed in this work the prehydrolyzates derived from a series of experiments aiming at the optimization of steam explosion pretreatment of beechwood [24] for their content of inhibitors and quantified their inhibitory effect on the enzymatic hydrolysis of Avicel as a model of a microcrystalline cellulosic substrate.

2. Materials and Methods

2.1. Biomass Feedstock

In this study, beechwood (*Fagus sylvatica*) harvested in winter 2015/2016 from a forest in Messen, canton Solothurn, Switzerland, was used as feedstock. It was it was chopped to a particle size of G30 air-dried to a final dry matter of 94% and milled (Retsch, SM 100, Haan, Germany) through a screen to a particle size of <1.5 mm. The untreated beechwood contained 40.8 ± 1.2% glucan, 19.1 ± 0.6% xylan, 25.3 ± 0.9% acid insoluble lignin, 7.3 ± 0.2% acetyl, 0.5% ash and 0.9% extractives as measured according to Sluiter et al. [25].

2.2. Steam Explosion Pretreatment

All steam explosion pretreatment experiments were performed with a custom-built steam gun system (Industrieanlagen Planungsgesellschaft m.b.H., Graz, Austria), as described in the companion publication [24]. Briefly, we performed a series of pretreatment experiments with severities (log R_0) ranging from 3.0 to 5.25 at reaction temperatures between 160 and 230 °C using 250 g of air-dried beechwood per run (Table 1). The severity was calculated according to Overend et al. based on the pretreatment time t [min] and the pretreatment temperature T [°C] [26]:

$$\log R_0 = \log\left(t \cdot e^{\frac{T-100}{14.75}}\right) \quad (1)$$

Table 1. Summary of the tested pretreatment conditions. Shown are the pretreatment durations in [min] to reach the targeted severities log R_0 (see Equation (1)) at a given temperature.

Temperature [°C]	Pretreatment Severity log R_0									
	3	**3.25**	**3.5**	**3.75**	**4**	**4.25**	**4.5**	**4.75**	**5**	**5.25**
160	17.1	30.4	54.1	96.2	171.2	-	-	-	-	-
170	-	15.5	27.5	48.9	86.9	154.5	-	-	-	-
180	-	-	13.9	24.8	44.1	78.4	139.5	-	-	-
190	-	-	-	12.6	22.4	39.8	70.8	125.9	-	-
200	-	-	-	6.4	11.4	20.2	35.9	63.9		-
210	-	-	-	-	5.8	10.3	18.2	32.4	57.7	-
220	-	-	-	-	2.9	5.2	9.3	16.5	29.3	-
230	-	-	-	-	-	2.6	4.7	8.4	14.9	26.4

The pretreatment slurry was vacuum-filtered through Whatman No. 1 filter paper and the liquid phase was analyzed for its composition by HPLC and employed as reaction media in the enzymatic hydrolysis of Avicel as described below. Pretreatments were performed as single experiments, but one condition (log R_0 = 5.0, 230 °C) was performed in triplicates in order to estimate the reproducibility of the results. For this condition, the mean values together with the standard deviation of the mean are reported.

2.3. Enzymatic Hydrolysis

To test the inhibitory effect of the steam explosion prehydrolyzates independently from the characteristics of the pretreated solids, they were employed as part of the liquid reaction phase in the enzymatic hydrolysis of microcrystalline Avicel PH-101 (Sigma-Aldrich, Buchs, Switzerland) at a glucan concentration of 3.5% *w*/*w*. The amount of prehydrolyzate added to the reaction mixture was calculated to correspond to an identical amount as in the whole pretreatment slurry with a 3.5% *w*/*w* glucan concentration based on the composition of the untreated raw material. Due to excessive condensation of steam in long pretreatment runs, some pretreatment slurries had a glucan content lower than 3.5% *w*/*w* and were thus not utilized for the inhibition experiments. Other conditions resulted in such small amounts of condensate that the separation of a sufficient amount of prehydrolyzate for inhibition experiments was not possible. Prior to enzymatic hydrolysis, the pH of the prehydrolyzates was adjusted to a value of 4.8 using 1 M NaOH. Accellerase 1500 (DuPont, St Joseph, MO, USA) with an activity of 58.7 filter paper units (FPU)/mL and a protein content of 92 mg/mL was used as cellulase. In one experimental series, the xylanase mixture Accellerase XY (DuPont, St Joseph, MO, USA) was supplemented at a loading of 0.05 mL/g_{glucan}. Enzymatic hydrolysis experiments were performed in 0.05 M citric acid buffer (pH 4.8) employing a cellulase loading of 15 FPU/g_{glucan} or 24 mg protein/g_{glucan} at 50 °C for 168 h. Sodium azide (10 mg/L) was added to prevent microbial contamination.

The control experiment without the addition of hydrolysate was performed as duplicate. Here, the standard deviation of the glucose concentration after 24 h was 0.07 g/L (0.4%) and 0.3 g/L (0.8%) after

168 h. Due to the small standard deviation, all other enzymatic hydrolysis reactions were performed as single runs.

2.4. Multiple Linear Regression

To correlate the inhibitor concentrations with the glucose yield reduction in enzymatic hydrolysis, we performed a multiple linear regression with the concentration of the phenolics and the concentration of the xylose equivalents (i.e., the sum of the xylose and xylooligomer concentration) as independent variables using RStudio (Version 1.2.5033). The vertical intercept was set to zero and the zero-point was included as a data point in the dataset used for multiple linear regression.

2.5. Analytical Methods

Glucose, cellobiose, xylose, HMF, furfural, formic acid and acetic acid were quantified by HPLC (Waters 2695 Separation Module, Waters Corporation, Milford, CT, USA) and refractive index detection (Waters 410) using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) at 60 °C with 5 mM H_2SO_4 as the mobile phase flowing at 0.6 mL/min. The relative standard deviation of samples injected in triplicates was for all compounds below 0.25%, thus inhibitors were routinely quantified in single runs.

Xylooligomers were quantified following a dilute acid hydrolysis procedure as described by Sluiter et al. [27]. These runs were performed in triplicates and reported are the mean values. The mean standard deviation for xylooligomer concentration of all triplicates was 1.1% and the maximal standard deviation amounted to 3.5%. For a better readability, the corresponding error bars are not included in Figure 1.

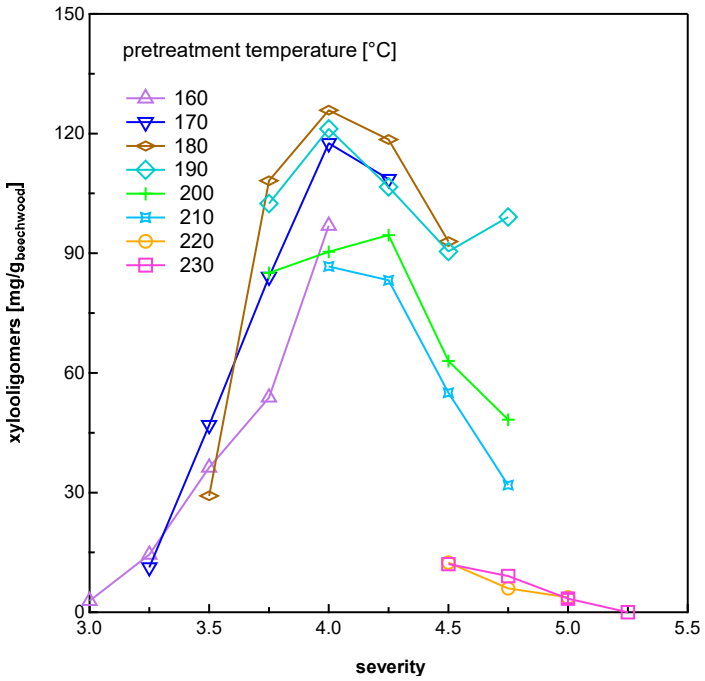

Figure 1. Release of soluble xylooligomers during steam explosion pretreatment. Shown are the amounts of soluble xylooligomers that are released into the prehydrolyzate during steam explosion pretreatment as function of the pretreatment severity and temperature.

The concentration of the total phenolics was determined after derivatization with the Folin–Ciocalteau reagent with a downscaled protocol based on Kapu et al. [28]. Hydrolysate samples were diluted 1:25 and 40 µL of the diluted sample was mixed with 100 µL Folin–Ciocalteau reagent (Sigma Aldrich). After 5 min, 300 µL of a 20% Na_2CO_3 solution was added, followed by the addition of 1560 µL water. The mixture was incubated for 2 h at 22 °C in an Eppendorf vial shaker, and then the

absorbance was measured spectrophotometrically at 760 nm. A 0.226 g/L gallic acid stock solution was used to build a standard curve with 5 dilutions. The standard deviation of the phenolics concentration for samples run in triplicates amounted to 4.8%.

3. Results and Discussion

3.1. Inhibitor Quantification

To understand the influence of the steam explosion pretreatment conditions on the extent of inhibition of enzymatic hydrolysis by the prehydrolyzates, we first quantified the most common inhibitory substances in the liquid phases derived from pretreatments of beechwood at severities between 3.0 and 5.25 and temperatures of 160–230 °C (Table 1). In steam explosion pretreatment, the reaction temperature is controlled by direct injection of saturated steam. During the reaction, the steam condenses and the prehydrolyzate is formed. The volume of prehydrolyzate depends on the pretreatment condition and varied in this experimental series from 184 to 5065 mL. Thus, inhibitor concentrations cannot be compared directly, and we normalized the results with respect to the amount of raw dry beechwood that went into pretreatment.

3.1.1. Pentose Sugars

In steam explosion and hot water pretreatment, xylan is solubilized by autohydrolysis catalyzed through the release of weak organic acids from the biomass [29]. The resulting sugar fraction consisting of soluble xylooligomers and monomeric xylose is an important intermediate product, but it also inhibits enzymatic hydrolysis and has to be taken into account in inhibitor studies [30]. During steam explosion pretreatment of beechwood, the highest amounts of xylooligomers were released at a severity of 4.0 at temperatures between 170 and 190 °C reaching a maximal value of 125 $mg/g_{beechwood}$ (Figure 1).

This corresponds to a yield of 66% of the total xylan present in the untreated biomass. At higher temperatures and severities, the amount of xylooligomers decreased, because they are completely hydrolyzed to xylose or degraded further to furfural. At lower severities, less xylooligomers could be found, because a higher part of the xylan fraction remained in the solid phase, as detailed in the companion paper. Overall, the severity factor is a poor predictor of the amount of solubilized xylan. At a given severity, the amount of xylooligomers is markedly influenced by the chosen pretreatment temperature. Similar observations have been reported by Kim et al. who introduced an altered severity factor that takes into account a higher influence of the reaction temperature [31]. However, as the common severity factor is widely known and accepted, we did not attempt to recalculate the severity factor.

Monomeric xylose was present at lower concentrations than the oligomers with a maximal value of 30 $mg/g_{beechwood}$ reached at a severity of 4.5 at a pretreatment temperature of 180 °C (Figure 2). Compared to the xylooligomer release, the monomeric xylose concentration reached the maximum at higher severities.

The maximal total amount of solubilized xylan (the sum of soluble xylooligomers and xylose) recovered in the liquid phase was 150 $mg/g_{beechwood}$ and was reached at a temperature of 180 °C with severities of 4.0 and 4.25. Here, monomeric xylose contributed only 4.5% and 10.2%, respectively, to the total amount of solubilized xylan. This is typical for hydrothermal pretreatments and is confirmed by Nitsos et al., who found in LHW pretreatment of beechwood a maximal total xylose concentration at a severity of 3.81 with monomeric xylose accounting for 9.5% of the total [32].

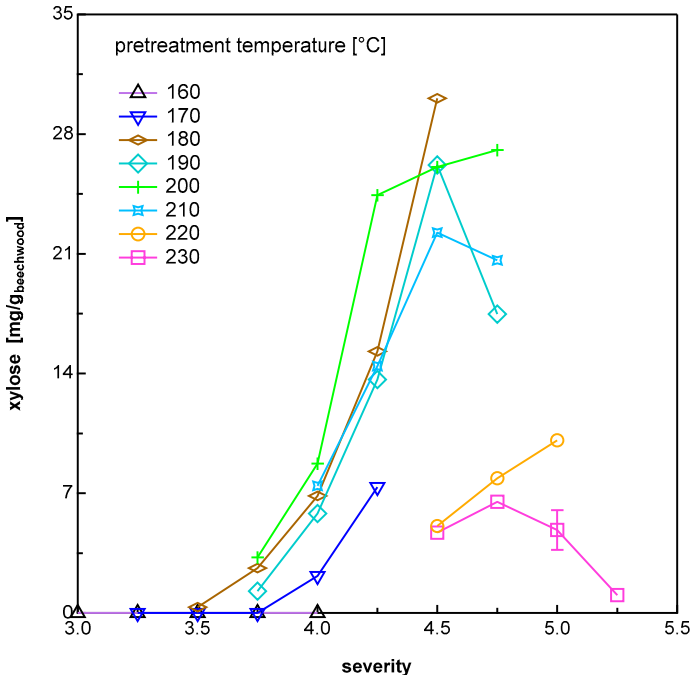

Figure 2. Formation of soluble xylose during steam explosion pretreatment. The amount of xylose that is released into the prehydrolyzate during steam explosion pretreatment is shown as a function of the pretreatment severity and temperature.

3.1.2. Phenolics

Phenolic compounds are formed during the pretreatment of biomass by partial solubilization of the lignin fraction of the biomass [33,34]. Especially the splitting of the labile β-*O*-4 ether bonds leads to wide range of soluble phenolics, such as 4-hydroxybenzoic acid, vanillin or syringaldehyde. A further possible source of phenolics are the extractives of the biomass that may contain for example gallic acid [10].

In this work, we did not separate the phenolic compounds, but measured the total phenolics concentration using the Folin–Ciocalteau reagent. The amount of released phenolics increased linearly with increasing pretreatment severity and independently of the pretreatment temperature and reached a maximum value of 21.6 mg/g$_{beechwood}$ at a severity of 5.25 (Figure 3).

In LHW pretreatment of maple at 200 °C for 20 min (log R_0 = 4.25), Kim et al. measured a phenolics concentration of 1.3 g/L, corresponding to an amount of 5.65 mg/g$_{maple}$, which is about 50% less than the value we found at that condition [17]. For LHW pretreatment of oil palm mesocarp fiber, the amount of generated phenolics ranged from 12 to 45 mg/g$_{biomass}$ for pretreatments at 150–220 °C and at severities of 3.25–4.83, thereby exceeding the values found in this study [35]. Even higher values of 55 and 66 mg/g$_{biomass}$ were recently reported for a low and a high lignin variant of LHW pretreated sugar cane bagasse (190 °C, 20 min, log R_0 = 3.95) [36].

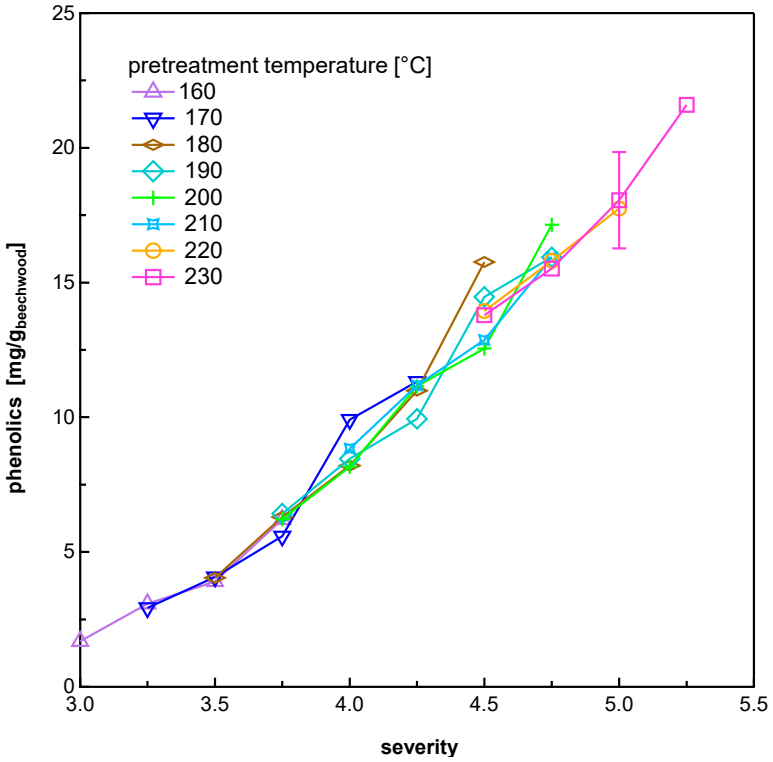

Figure 3. Formation of phenolics during steam explosion pretreatment. The amount of phenolics that is released into the prehydrolyzate during steam explosion pretreatment is shown as a function of the pretreatment severity and temperature.

3.1.3. Organic Acids

The hemicellulosic fraction of lignocellulosic biomass consists of heteropolysaccharides that are typically decorated with acetyl groups that are released during pretreatment through hydrolysis [37]. In our experiments, the amount of acetic acid in the prehydrolyzate increased exponentially with increasing severity up to a severity factor of 4.5. At higher values, the increase is linearly proportional to the severity factor (Figure 4). Correspondingly, the highest amount of acetic acid (43.7 mg/$g_{beechwood}$) was found at the highest tested severity of 5.25.

In liquid hot water pretreatments, more acetic acid was measured in the prehydrolyzate. For example, Kim et al. reported an acetic acid release of 57 mg/$g_{biomass}$ at a severity of 4.25 and Nitsos et al. found approximately 53 mg/$g_{biomass}$ acetic acid at a severity of 4.25 [17,32]. For a LHW pretreatment severity of 4.83, 75 mg/$g_{biomass}$ acetic acid were measured in the prehydrolyzate derived from oil palm mesocarp fibers [35]. It is possible that the acetic acid concentration is lower in steam explosion pretreatment, as it is a relatively volatile compound and thus may partly be evaporated after the explosive discharge of the biomass.

Formic acid can be formed during the pretreatment process as a decomposition product of furan aldehydes. During steam explosion pretreatment of beech, the reaction temperature had a significant influence on the formation of formic acid (Figure 5). At 200 °C and lower, only low concentration of formic acid of up to 5.6 mg/$g_{beechwood}$ could be found. At higher temperatures, formic acid concentration ranged from 6.2 to 10.9 mg/$g_{beechwood}$, reaching the maximum at the highest tested severity of 5.25. We also observed that the formic acid concentration decreased in some cases with increasing pretreatment severity, which suggests that formic acid is prone to further decomposition reactions.

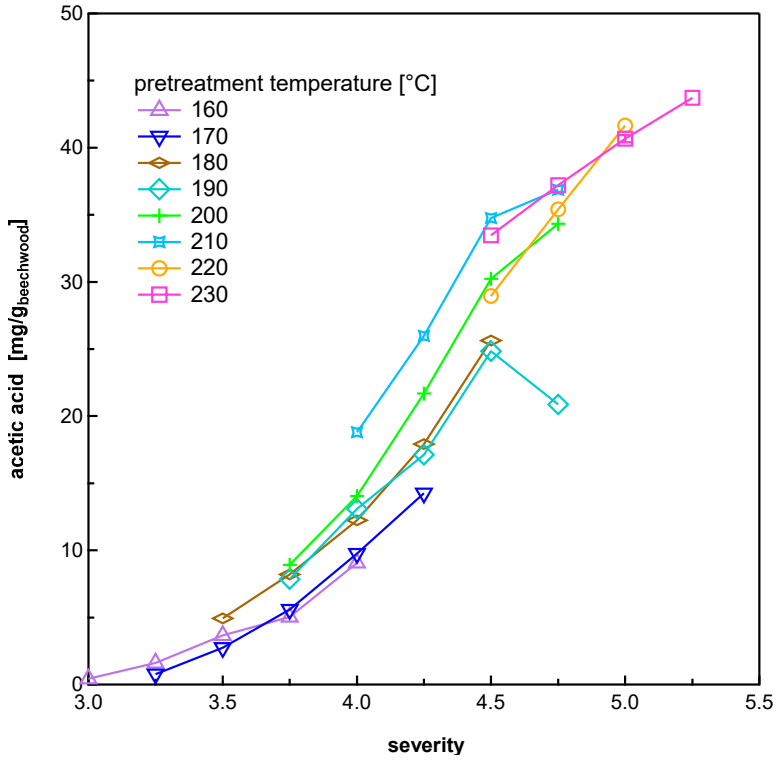

Figure 4. Formation of acetic acid during steam explosion pretreatment. The amount of acetic acid that is released into the prehydrolyzate during steam explosion pretreatment is shown as a function of the pretreatment severity and temperature.

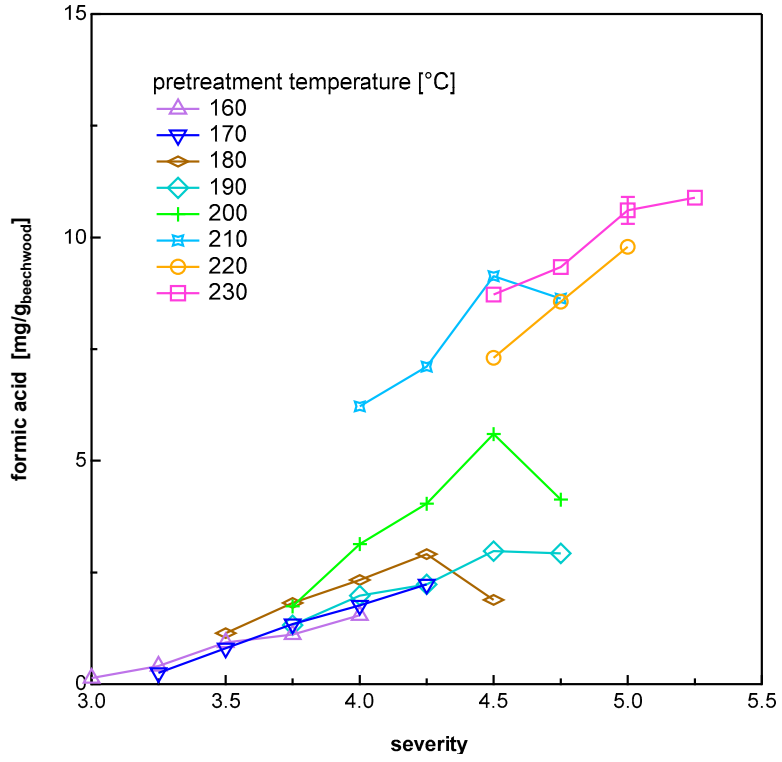

Figure 5. Formation of formic acid during steam explosion pretreatment. The amount of formic acid that is released into the prehydrolyzate during steam explosion pretreatment is shown as a function of the pretreatment severity and temperature.

Compared to a wet oxidation pretreatment of wheat straw, where 58 mg/$g_{biomass}$ (or 3.5 g/L at a solids concentration of 60 g/L) formic acid were detected, our values are about five times lower [38]. As with acetic acid, we assumed that part of the formic acid evaporated during the explosive pressure release.

3.1.4. Furan Aldehydes

The furan aldehydes, HMF and furfural, are sugar degradation products that are derived from hexoses (HMF) or pentoses (furfural), respectively. As the hemicellulosic fraction of beech mainly contained xylose and only a negligible part of glucan is solubilized during pretreatment, the concentration of HMF was low at severities smaller than 4.25 and did not exceed a value of 1 mg/$g_{beechwood}$ (Figure 6). With increasing severity, the concentration of HMF increased exponentially up to a maximal concentration of 8.3 mg/$g_{beechwood}$. At identical severities, we observed the tendency that higher reaction temperatures favor the formation of HMF.

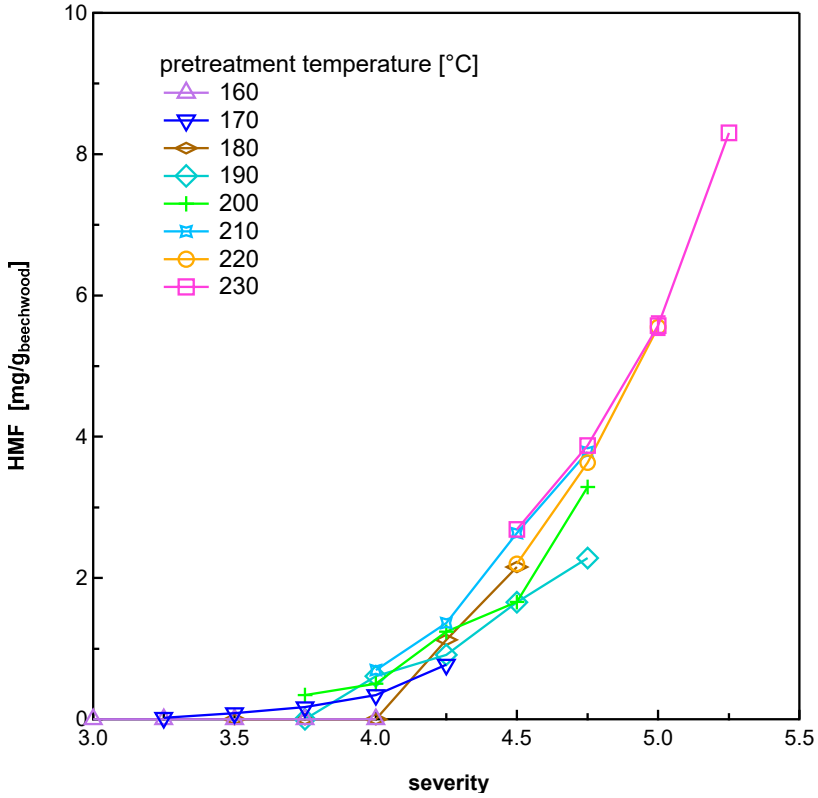

Figure 6. Formation of HMF during steam explosion pretreatment. The amount of HMF that is released into the prehydrolyzate during steam explosion pretreatment is shown as a function of the pretreatment severity and temperature.

The amount of the xylose degradation product furfural formed during steam explosion pretreatment of beech was markedly influenced by the reaction temperature (Figure 7). The maximal furfural concentration of 9.2 mg/$g_{beechwood}$ was detected at a pretreatment severity of 4.75 at a temperature of 200 °C. At higher temperatures, the amount of furfural decreased, for example the furfural concentrations at the 230 °C pretreatment were lower than the ones found at the 220 °C pretreatments. The reason for this observation is likely the thermal instability of furfural, as it can react further to formic acid [10].

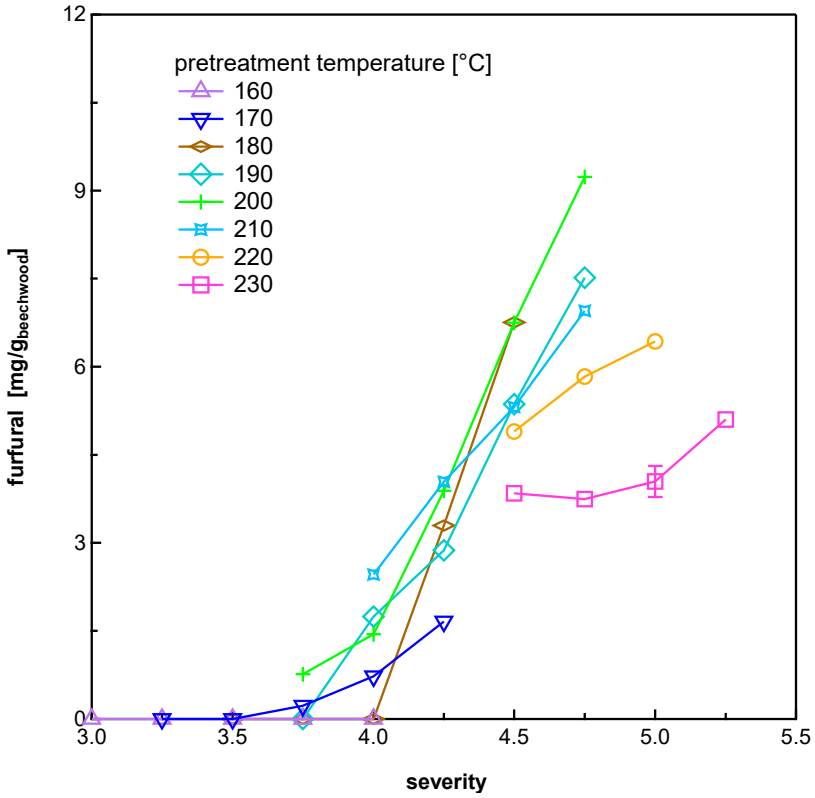

Figure 7. Formation of furfural during steam explosion pretreatment. The amount of furfural that is released into the prehydrolyzate during steam explosion pretreatment is shown as a function of the pretreatment severity and temperature.

The total amount of furan aldehydes released during steam explosion pretreatment is lower than during hot water pretreatment of maple. Kim et al. reported a value of 18 mg/g$_{biomass}$ at a severity of 4.24 and 200 °C [17], while we found at identical conditions only about 5.5 mg/g$_{beechwood}$ HMF and furfural.

3.2. Inhibition of Enzymatic Hydrolysis

To quantify the inhibitory effect of the different prehydrolyzates, we used them as part of the liquid fraction in the enzymatic hydrolysis of microcrystalline cellulose. Enzymatic hydrolysis was run at a glucan concentration of 3.5% and the amount of prehydrolyzate added corresponded to the amount that would be present in a whole slurry enzymatic hydrolysis of steam exploded beechwood at a similar glucan concentration. The concentrations of the inhibitors in the enzymatic hydrolysis reaction mixture is presented in Table S2. The reactions were sampled after 24 and 168 h.

All prehydrolyzates reduced the final glucose yield, albeit to a different extent (Figure 8). Depending on the pretreatment conditions, yields were reduced by 5–26% compared to the control. The most inhibiting prehydrolyzate derived from the pretreatment with the highest severity of 5.25 at a pretreatment temperature of 230 °C. The extent of the inhibition depended not only on the pretreatment severity, but also on the pretreatment temperature. At a severity of 3.5, the glucose yield was decreasing with increasing pretreatment temperature, while at a severity of 4.75 the trend is vice versa. Especially the presence of the 200 °C prehydrolyzates (covering a severity range from 3.75 to 4.5) resulted in an enhanced enzyme inhibition, with the extent being comparable to the effect of the prehydrolyzates derived at a severity of 5.0 and temperatures of 220 and 230 °C.

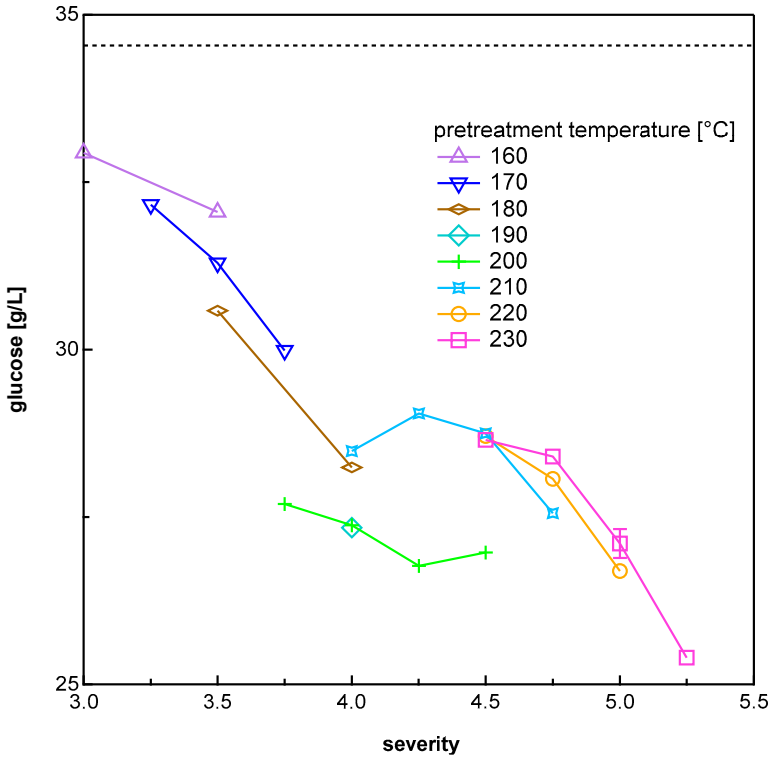

Figure 8. Effect of inhibitors in the steam explosion pretreatment hydrolysates on the enzymatic hydrolysis of Avicel. Shown are the final glucose concentrations after 168 h in the enzymatic hydrolysis of Avicel in the presence of different steam explosion prehydrolyzates as a function of pretreatment severity and temperature. The dashed horizontal line denotes the glucose concentration in the control without prehydrolyzate.

When analyzing the initial hydrolysis yields after 24 h (Figure 9), it could be determined that at this point in the hydrolysis reaction the inhibition was in most cases higher than at the final sample point (Table S2). Only for the pretreatments at a temperature of 230 °C or at the severity of 3.0, the final yield reduction was slightly higher than after 24 h. The latter cases may point to a phenolics induced deactivation of the cellulolytic enzymes, as reported by Ximenes et al. [39], who distinguished between reversible enzyme inhibition (occurring from the start of the reaction) and irreversible enzyme deactivation (that reduces enzyme activity especially at longer reaction times). For all other pretreatment conditions, the initial yield reduction was by a factor of 1.01–2.96 higher than the final yield reduction (see Table S2, where the ratio between the yield reduction after 24 and 168 h is shown). We hypothesized that this phenomenon is related to the amount of xylooligomers present in the hydrolysate. At prolonged hydrolysis times, the xylooligomers are hydrolyzed to xylose, which is known to have a weaker inhibitory effect than the oligomers [30,40]. In line with this explanation is also the observation that, at pretreatment temperatures of 200 and 210 °C, the initial glucose concentration is increasing with increasing severity (Figure 9). At these pretreatment temperatures, the xylooligomer amount is decreasing with increasing pretreatment severity (Figure 1).

To investigate the influence of the xylooligomers on the 24 h glucose concentrations, we subjected the 210 °C prehydrolyzates to a dilute acid hydrolysis prior to the enzymatic hydrolysis. In this step, all xylooligomers were converted to xylose. Alternatively, we added xylanase to the enzyme cocktail which had a similar effect. In both cases, the 24 h glucose yields could be improved, and the degree of inhibition was constant over the range of the tested severities (Figure 10). Sampling at 168 h showed that the final glucose yields were not improved by the xylanase addition, thus the increased yield after 24 h could be attributed to the xylooligomer hydrolysis.

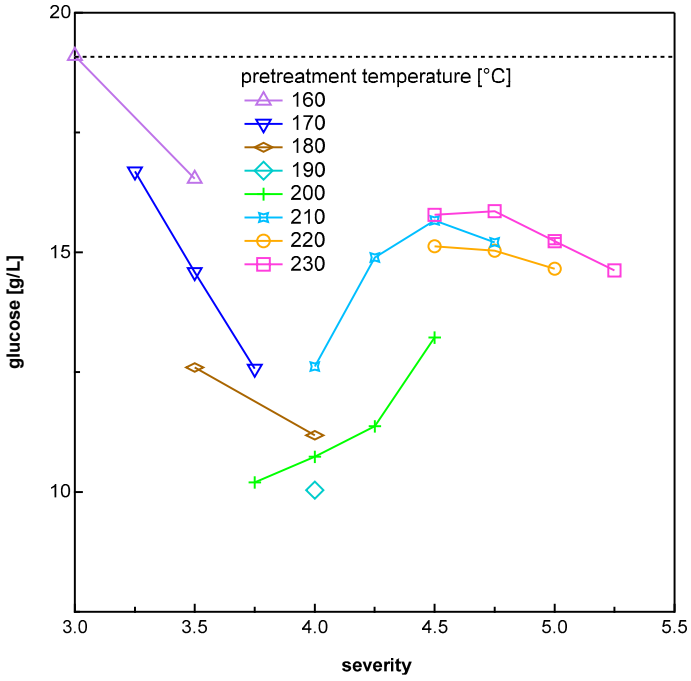

Figure 9. Effect of inhibitors in the steam explosion pretreatment hydrolysates on 24 h glucose yield during enzymatic hydrolysis of Avicel. Shown are the glucose concentrations after 24 h in the enzymatic hydrolysis of Avicel in the presence of different steam explosion prehydrolyzates as a function of pretreatment severity and temperature. The dashed horizontal line denotes the glucose concentration in the control without prehydrolyzate.

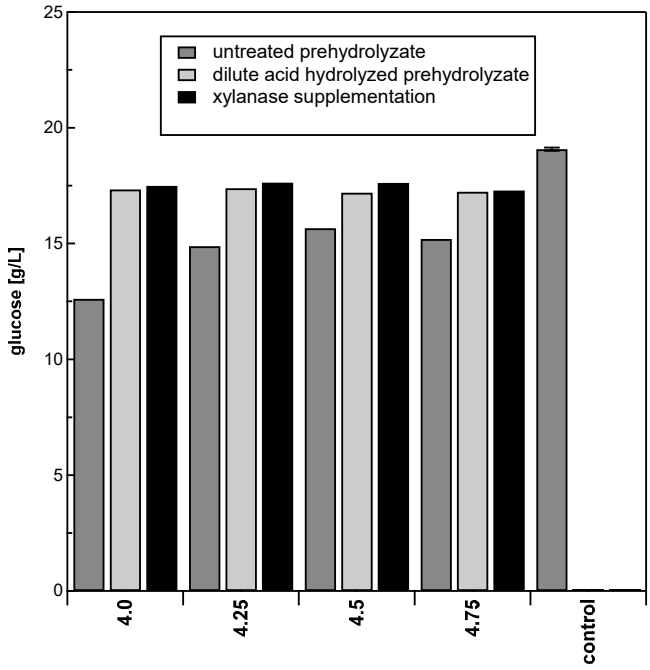

Figure 10. Initial glucose concentrations (24 h) in the enzymatic hydrolysis of Avicel in the presence of 210 °C steam explosion prehydrolyzates: effect of xylanase supplementation and acid hydrolysis of xylooligomers. The dark grey bar on the right site of the graph denotes the glucose concentration in the control without prehydrolyzate. One group of prehydrolyzates was subjected to a dilute acid hydrolysis to convert xylooligomers to xylose (light grey bars). Alternatively, xylanase was added to the enzymatic hydrolysis mixtures (black bars).

3.3. Relation between Inhibitor Concentrations and Extent of Enzyme Inhibition

Based on the available dataset, we attempted to relate the measured inhibitor concentrations with the extent of the glucose yield reduction in enzymatic hydrolysis. As a starting point, we first investigated how the glucose yield in enzymatic hydrolysis is influenced if the concentration of one specific prehydrolyzate (230 °C, log R_0 = 5.0) is altered. In the tested concentration range, a linear correlation could be found. With a higher prehydrolyzate concentration, the observed yield reduction increased (Figure S1). Consequently, we assumed that a multiple linear regression approach would be valid to predict the yield reduction in enzymatic hydrolysis as function of the inhibitor concentrations. However, a dataset of 23 experiments is too small for linear regression with six different inhibitor concentrations as variables. Thus, we aimed at reducing the number of variables by selecting only the most important inhibitors. To this end, we sorted the data for decreasing yield reduction. Among the eight prehydrolyzates that led to a final yield reduction of more than 20%, there were four prehydrolyzates that contained the four highest amounts of phenolics and three prehydrolyzates that ranked among the top four regarding the amount of xylose equivalents (i.e., the sum of the xylose and xylooligomer concentration). We did not distinguish between monomeric xylose and xylooligomers, because the latter are depolymerized over the course of the enzymatic hydrolysis reaction and therefore influence primarily the initial hydrolysis rates that are not part of this analysis. The observation that the total phenolics and xylose concentrations are most relevant is in accordance to the available literature data. For instance, Zhai et al. made a similar observation and concluded that the sugars in the SO_2 catalyzed steam explosion prehydrolyzate were responsible for about 60% of the inhibition of the hydrolytic enzymes and the phenolics for around 40% of the yield reduction [22]. HMF, furfural and acetic acid in concentrations typical for hydrolysates were found to be hardly influencing the enzymatic hydrolysis of lignocellulose [17,22], but elevated concentrations of formic acid are disadvantageous. For example, the presence of 4 g/L of formic acid reduced glucan hydrolysis by 20% and the enzymes were completely inactivated at a concentration of 15 g/L [41]. In our experiments, however, the formic acid concentrations never exceeded 1 g/L, which is not expected to influence the enzymatic hydrolysis much.

Consequently, we performed a multiple linear regression to predict the final yield reduction in enzymatic hydrolysis of Avicel as a function of only the phenolics concentration ($c_{phenolics}$) and the sum of the concentrations of xylose and xylooligomers ($c_{xylose\ eq.}$) in the enzymatic hydrolysis mixtures (see Table S2), as expressed in the following equation:

$$Enzymatic\ hydrolysis\ yield\ reduction\ [\%] = P{\cdot}c_{phenolics} + X{\cdot}\ c_{xylose\ eq.} + 0 \quad (2)$$

A significant regression equation was found ($p < 2.2 \times 10^{-16}$) with an adjusted r^2 of 0.9805. The coefficients are P = 13.2 ± 0.6 L/g and X = 0.8 ± 0.1 L/g.

A three-dimensional plot of Equation (2) together with the measured data points is shown in Figure 11.

The relative inhibitory effect of the phenolics is more pronounced than the one of the xylose equivalents. If the maximal amount of xylose equivalents we found in our dataset (12.87 g/L) were the only inhibitor, the enzymatic hydrolysis yield would be reduced by 10.3%, while, in the presence of the maximal concentration of phenolics (1.85 g/L), the yield reduction would amount to 24.4%. For the investigated dataset, the phenolics contributed 46–100% to the total calculated yield reduction and were dominating in most cases (see Table S2).

The first part of this two-paper series showed that a reaction temperature of 230 °C and high severities are necessary for maximal cellulose reactivity. Under these conditions however most of the hemicelluloses are destroyed, which points to the need of a two-stage pretreatment process, where Stage 1 will take place at 180–200 °C to recover solubilized xylan and Stage 2 will be performed at 230 °C. Based on the present inhibitor study, we predict that such a combined hydrolysate would show a high degree of inhibition to enzymatic hydrolysis because under such conditions the amounts

of both xylose and phenolics are high. Consequently, whole slurry enzymatic hydrolysis of the solids should be performed without the first stage prehydrolyzate to avoid inhibition by the C5 sugars. This hypothesis will be tested in future experimental work.

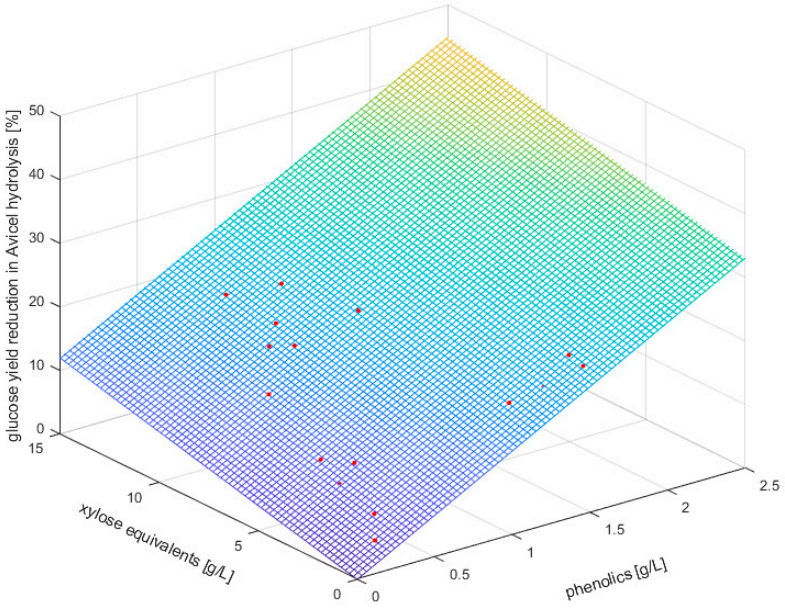

Figure 11. Calculated final yield reduction in enzymatic hydrolysis of Avicel as a function of the concentration of the sum of xylose and xylooligomers and of the phenolics concentration in the enzymatic hydrolysis mixture. Shown is the calculated inhibition plane based on Equation (2) together with the measured data points in red.

4. Conclusions

In this work, we investigated how the reaction conditions in steam explosion pretreatment of beechwood with severities ranging from log R_0 = 3.0 to 5.25 at reaction temperatures between 160 and 230 °C influenced the resulting amount of different enzyme and fermentation inhibitors. We found that the amount of phenolics, HMF and acetic acid were increasing with increasing pretreatment severity independently of the reaction temperature. On the other hand, the formation of formic acid was markedly increased at reaction temperatures of 210 °C and above, while furfural and soluble C5 sugars concentrations peaked at intermediate severities.

The presence of the different inhibitor mixtures in enzymatic hydrolysis of Avicel lowered the final glucose yields by 5–26% at a glucan loading of 3.5%. Mainly, the amount of phenolics and xylose equivalents were contributing to the reduced yield.

Future work will investigate the option of two stage steam explosion treatment and the inhibition of the fermentation step by the different prehydrolyzates, with the aim to find a route that allows for whole slurry processing of pretreated biomass to the final fermentation product without any washing and detoxification steps.

Supplementary Materials: The following file is available online at http://www.mdpi.com/1996-1073/13/14/3638/s1, Figure S1: Influence of the amount of prehydrolyzate (230 °C, log R_0 = 5.0) on the yield reduction in the enzymatic hydrolysis of Avicel at 3.5% solids concentration, Figure S2: Final glucose concentrations (168 h) in the enzymatic hydrolysis of Avicel in the presence of 210 °C steam explosion prehydrolyzates: effect of xylanase supplementation and acid hydrolysis of xylooligomers, Table S1: Selected literature data on inhibitors generation in different kinds of pretreatment and their influence on enzymatic hydrolysis, Table S2: Summary of inhibitor concentrations in the enzymatic hydrolysis mixture and their effect on enzymatic hydrolysis of Avicel.

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