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Combined Use of H_2SO_4 and SO_2 Impregnation for Steam Pretreatment of Spruce in Ethanol Production

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Abstract

Fuel ethanol can be produced from softwood through hydrolysis in an enzymatic process. Prior to enzymatic hydrolysis of the softwood, pretreatment is necessary. In this study, two-step steam pretreatment employing dilute H_2SO_4 impregnation in the first step and SO_2 impregnation in the second step, to improve the overall sugar and ethanol yield, was investigated. The first pretreatment step was performed under conditions of low severity (180°C, 10 min, 0.5% H_2SO_4) to optimize the amount of hydrolyzed hemicellulose. In the second step, the washed solid material from the first pretreatment step was impregnated with SO_2 and pretreated under conditions of higher severity to make the cellulose more accessible to enzymatic attack, as well as to hydrolyze a portion of the cellulose. A wide range of conditions was used in the second step to determine the most favorable combination. The temperatures investigated were between 190 and 230°C, the residence times were 2, 5, and 10 min; and the SO_2 concentration was 3%. The effect of pretreatment was assessed by both enzymatic hydrolysis of the solids and by simultaneous saccharification and fermentation (SSF) of the whole slurry, after the second pretreatment step. For each set of pretreatment conditions, the liquid fraction was also fermented to determine any inhibitory effects. Ethanol yield using the SSF configuration reached 66% of the theoretical value for pretreatment conditions in the second step of 210°C and 5 min. The sugar yield using the separate hydrolysis and fermentation configuration reached 71% for pretreatment conditions of 220°C and 5 min.

Index Entries: Enzymatic hydrolysis; softwood; simultaneous saccharification and fermentation; separate hydrolysis and fermentation.

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Introduction

Acid-catalyzed steam pretreatment of softwood is an effective way of increasing the overall yield in the wood-to-ethanol process. This form of pretreatment both increases the recovery of carbohydrates and enhances the enzymatic hydrolysis *(1)*. SO_2 and H_2SO_4 are two acids that have been used as catalysts.

It is well known that more severe conditions during steam pretreatment will cause greater degradation of hemicellulose sugars *(2–5)*. However, a high degree of severity is required to enhance the enzymatic digestibility of the cellulose fibers, especially in softwood *(6)*. Steam pretreatment causes the hemicellulose to be degraded to its monomeric sugars and the structure of cellulose to soften to make it more accessible to enzymes in the following steps. However, the maximum yields of hemicellulose sugars and glucose from cellulose are not reached at the same degree of severity in the pretreatment. If a high degree of severity is used sugars may degrade further to furfural, 5-hydroxymethylfurfural (HMF), levullinic acid, and formic acid together with other substances. The formation of degradation products reduces the overall yield, and the products may also cause inhibition in downstream process steps. On the other hand, if a low degree of severity is used, cellulose digestibility will not be enhanced, which will cause the overall sugar yield to be lower.

The overall yield has been found to be the most important parameter when evaluating the production cost of bioethanol *(1)*. Since the highest costs in the process are those of the raw material *(2)* and the enzymes, it is important to ensure a high degree of utilization of the carbohydrate components in the feedstock to decrease production cost. The idea of a two-step steam pretreatment process has been proposed in the literature several times as a means of increasing overall yield *(4,6,7–10)*.

It has been shown that H_2SO_4 impregnation enhances the recovery of hemicellulose sugars following pretreatment, while impregnation with SO_2 promotes a higher recovery of glucose after enzymatic hydrolysis and also results in less inhibition in the fermentation step compared with H_2SO_4 *(11)*. This indicates that two-step steam pretreatment has the potential to result in higher sugar yields. Tengborg et al. *(11)* proposed two-step steam pretreatment in which the first step was performed at low severity with H_2SO_4 as the acid catalyst to hydrolyze the hemicellulose. In the second step, the washed solid material from the first step was impregnated with SO_2 and steam pretreated, this time at high severity, to enhance the enzyme accessibility *(11)*.

In the present study, we investigated a two-step steam pretreatment process. The conditions in the first pretreatment step were chosen to give a high recovery of fermentable hemicellulose sugars in the liquid, which involved the use of H_2SO_4 as the acid catalyst. The solid material in the slurry was washed with water and then treated again in the second pretreatment step. In the second step SO_2 was used as the acid catalyst to

promote cellulose degradation by enzymes in the subsequent step, irrespective of whether enzymatic hydrolysis or simultaneous saccharification and fermentation (SSF) was used. We focused on utilization of hexoses, in this case mainly mannose and glucose, since they can be fermented by *Saccharomyces cerevisae*, the yeast used in this study. The effect of pretreatment was assessed by both separate hydrolysis and fermentation (SHF) and by simultaneous SSF. The second pretreatment step was optimized with respect to the overall ethanol yield after SSF and, for SHF, to the overall yield of fermentable sugars after enzymatic hydrolysis.

Materials and Methods

Procedure

The experimental procedure employed is shown schematically in Fig. 1. The softwood was impregnated with dilute H_2SO_4 and then steam pretreated. The resulting material was separated into a solid residue and a liquid. The liquid was analyzed regarding sugars and then fermented. The solid material was washed with water, then impregnated with gaseous SO_2 and steam pretreated in the second pretreatment step. The resulting material was evaluated by SSF of the slurry, by enzymatic hydrolysis of the washed solid material, and by fermentation of the liquid.

The severity factor is often used for the evaluation of steam pretreatment. Although it does not provide an accurate measure of the severity, it can be used for rough estimates *(9)*. The severity correlation describes the severity of the pretreatment as a function of treatment time (min) and temperature (°C), in which T_{ref} = 100°C *(12)*:

$$\text{Log } Ro = \log\left(t \cdot \exp\left[\frac{T - T_{ref}}{14.75} \right] \right) \quad (1)$$

Raw Material

Fresh spruce, *Picea abies*, free from bark, was used. Sawdust was supplied by local sawmills. The composition was determined according to the Hägglund *(13)* method and is presented in Table 1. The raw material used for impregnation with H_2SO_4 in the first step had a dry matter (DM) content of 55.5%.

Pretreatment

First Pretreatment Step

The first steam pretreatment step was optimized and performed at the Mid Sweden University, Örnsköldsvik, in a 250-L batch reactor located in Rundvik, Sweden *(14)*. The reactor is a Masonite gun with direct steam injection. The heat-up time is 30 s. The sawdust was impregnated with dilute H_2SO_4 (0.5% [w/w] based on the water content of the wood) and pretreated at 180°C for 10 min. The reaction was quenched by blowing the

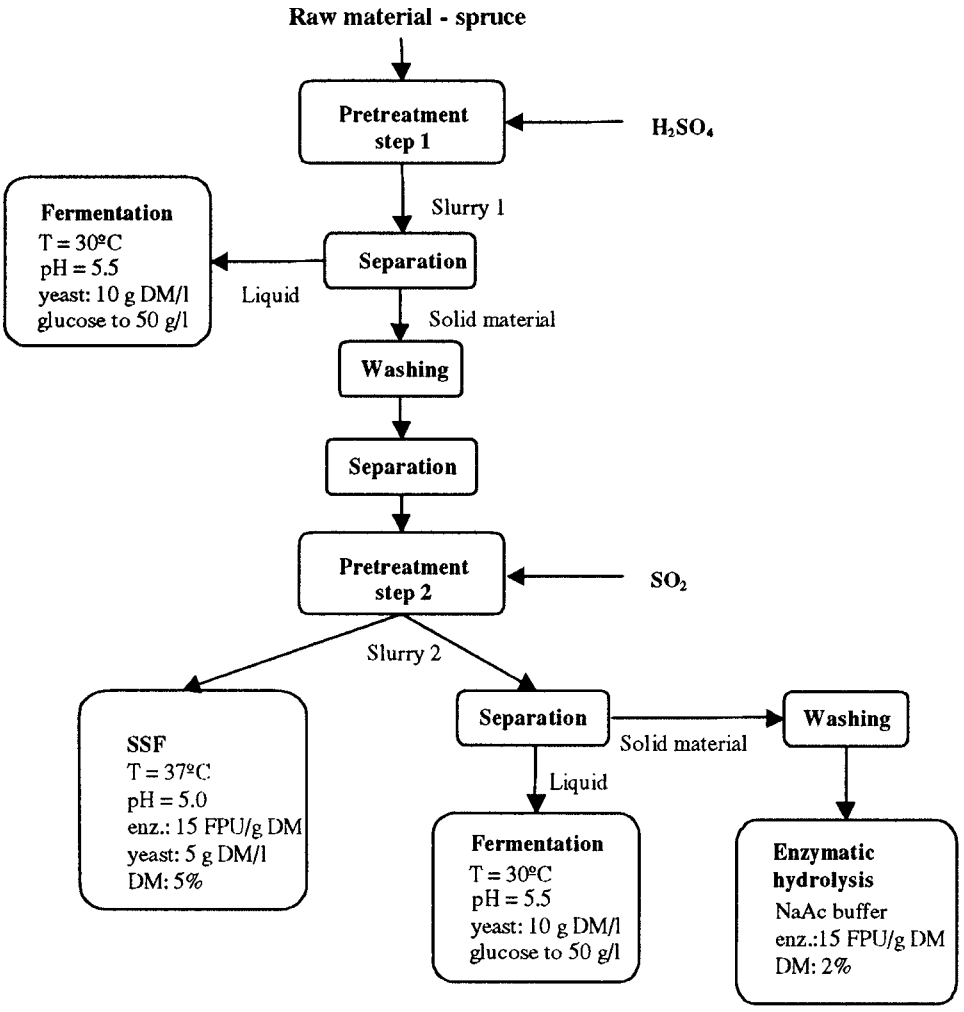

Fig. 1. Experimental setup used for evaluation of two-step steam pretreatment.

material to a flash tank at atmospheric pressure. The impregnated material had a DM content of 30%. The pretreated material, with a DM of 13%, was separated by centrifugation into a solid residue and a liquid. The liquid was analyzed regarding soluble sugars, and their degradation products. The composition of the solid material was determined with the Hägglund *(13)* method. The solid material was washed thoroughly with water to remove all soluble substances and the yield and composition of the solid material were determined *(13)*.

Second Pretreatment Step

The second steam pretreatment step was performed at Lund University in a steam-explosion unit with a 2-L reactor volume *(8)*. The solid washed material from the first pretreatment step, with a DM content of

Table 1
Composition of Raw Material and Material After First Pretreatment Step

Composition	Raw material (% of DM)	First-step material (% of DM)
Glucan	49.9	53.7
Mannan	12.3	2.1
Lignin	28.7	38.4
Xylan	5.3	1.6
Galactan	2.3	0
Arabinan	1.7	0.6

Table 2
Experimental Design of Second Pretreatment Step

Experiment	Tempature (°C)	Time (min)	Log (*R0*)
1	190	2	2.95
2	190	5	3.35
3	190	10	3.65
4	200	2	3.25
5	200	5	3.64
6	200	10	3.94
7	210	2	3.54
8	210	5	3.94
9	210	10	4.24
10	220	2	3.83
11	220	5	4.23
12	225	5	4.38
13	230	5	4.53

37%, was reimpregnated with gaseous SO_2. The material was placed in plastic bags for a 20-min impregnation at room temperature to reach a concentration of 3% SO_2 ([w/w], based on the water content of the wood). The impregnated material was steam pretreated in the second pretreatment step at various temperatures (190, 200, 210, 220, 225, and 230°C) and residence times (2, 5, and 10 min) (*see* Table 2). A portion of the pretreated material was separated by filtration into a solid residue and a liquid for evaluation with separate enzymatic hydrolysis and fermentation, and some was kept intact for evaluation with SSF. The liquid was analyzed with respect to soluble sugars and to their degradation products. The amount of insoluble solids in the pretreated material was determined.

In two cases, excessive washing was performed on the material from the first step to try to improve the SO_2 absorption in the impregnation prior to the second pretreatment step. Two pretreatment experiments, nos. 8 and 11, which resulted in the highest overall yields in SSF and SHF, respec-

tively, were repeated using this more thoroughly washed material. The temperatures used were 210 and 220°C, and a residence time of 5 min was used in both cases. SSF, enzymatic hydrolysis, and fermentation were performed as described earlier.

The equipment and the procedure for determination of oligosaccharides and for enzymatic hydrolysis, SSF, fermentation, and analysis have been described in more detail previously *(9)*. The methods are summarized next.

Determination of Oligosaccharides by Acid Hydrolysis

The amount of oligomers in the liquid after the first pretreatment step was determined by acid hydrolysis. This was performed in two ways: by autohydrolysis using the acetic acid present in the liquid, and by the addition of H_2SO_4.

Enzymatic Hydrolysis

Enzymatic hydrolysis of the washed solid material was used to assay the second pretreatment step. A commercial cellulase mixture, Celluclast 1.5L (65 filter paper units (FPU)/g and 17 IU/g of β-glucosidase) was used, supplemented with the β-glucosidase preparation Novozyme 188 (376 IU/g of β-glucosidase), both kindly donated by Novozymes A/S (Bagsværd, Denmark). Duplicate experiments with the washed solid material were performed. A DM concentration of 2% (w/w) was used to avoid end-product inhibition in determination of the potential sugar yield. A total of 10 g of DM, 2.32 g of Celluclast, and 0.52 g of Novozym were immersed in 0.1 mol/L of NaAc buffer to a total mass of 500 g. Hydrolysis was performed at 40°C and pH 4.8 for 96 h.

Simultaneous Saccharification and Fermentation

To assess the steam pretreatment conditions, SSF of the slurry from the second pretreatment step was used as an alternative method. The slurry was diluted with water to a final insoluble solids concentration of 5% DM. The cellulase activity was 15 FPU/g of DM and the β-glucosidase activity was 23 IU/g of DM, which was the same as in the enzymatic hydrolysis. Compressed baker's yeast, *S. cerevisiae* (Jästbolaget AB, Rotebro, Sweden) was used at an initial concentration of 5 g of DM/L. Antibiotics were added to prevent infection and the formation of lactic acid to ensure comparable results. SSF was performed at 37°C and pH 5.0 for 72 h. All experiments were performed in duplicate, and the average values are presented.

Fermentation

Fermentation of the liquid was performed after the first and the second pretreatment steps to investigate the fermentability and the extent of inhibition. Glucose was added to the liquids to adjust the concentration of fermentable sugar to 50 g/L. A reference solution containing 30 g/L of

glucose and 20 g/L of mannose was also fermented. *S. cerevisiae* was used at a concentration of 10 g of DM/L. Fermentation was performed at 30°C and pH 5.5 for 24 h. Experiments were performed in duplicate.

Analysis

The liquids after the pretreatment steps and all samples from the acid and the enzymatic hydrolysis, fermentation, and SSF were analyzed by high-performance liquid chromatography (HPLC) (Shimadzu LC-10AT; Shimadzu, Kyoto, Japan) with a refractive index detector (Shimadzu). Glucose, mannose, arabinose, galactose, and xylose were separated using an Aminex HPX-87P column (Bio-Rad, Hercules, CA) at 80°C, using water as the eluent, at a flow rate of 0.5 mL/min. Cellobiose, glucose, arabinose, lactic acid, glycerol, acetic acid, ethanol, HMF, and furfural were separated on an Aminex HPX-87H column (Bio-Rad) at 65°C using 5 mmol/L H_2SO_4 as the eluent, at a flow rate of 0.5 mL/min. All samples were filtered through a 0.20-µm filter before HPLC analysis. Samples from the enzymatic hydrolysis and the liquid phases after the pretreatment steps were analyzed on the HPX-87P column. However, because of interference between ethanol and mannose on that column, samples from SSF and fermentation were analyzed on the HPX-87H column. Analysis of glucose in the liquid phase after pretreatment was also carried out on the HPX-87H column.

Results and Discussion

First Pretreatment Step

The composition of the dry raw material is presented in Table 1. Sixty-two percent of the dry raw material consisted of glucan and mannan. The material used has also been used in a previous study, in which the results from the first pretreatment step have been discussed in detail *(10)*, and, hence, they are only summarized here. Ninety-three percent of the glucan was recovered after the first pretreatment step. Eighty-one percent was still present in the solids, whereas 12% was hydrolyzed and present in the liquid as either oligomeric or monomeric sugars. Thirteen percent of the solubilized glucan was recovered as oligomeric sugar. The recovery of mannan was 100%. Eighty-eight percent of the mannan was solubilized and found in the liquid, while the remainder was still in the nonhydrolyzed fibrous material. Some of the solubilized mannan (12%) was recovered as oligomeric sugars.

The liquid contained only small amounts of furfural and HMF: 0.7 and 1.4 g/L, respectively. Acetic acid was present at a concentration of 3.7 g/L. The amount of acetic acid, 1.6 g/100 g of dry raw material, corresponds well with the degree of acetyl substitution in galactoglucomannan.

Fermentation of the liquid from the first pretreatment step resulted in a yield of 0.48 g of ethanol/g of sugar, i.e., 94% of the theoretical fermentation yield (data not shown), which was the same as for the reference solution. The

productivity of ethanol was about half that of the reference solution after 4 h, but after 24 h the yield was the same as for the reference solution.

Second Pretreatment Step

The second pretreatment step was performed using the washed solid material from the first pretreatment step. This material contained mainly glucan (53.7%) and lignin (38.4%). Only small amounts of some of the hemicellulosic sugars were present: mannan (2.1%), xylan (1.6%), and arabinan (0.6%) (Table 1). The investigation covered a severity factor range of Log *Ro* from 2.95 to 4.53 (Table 2). The second pretreatment step was evaluated using SSF and enzymatic hydrolysis to determine the ethanol yield and the glucose yield, respectively.

Although impregnation with SO_2 in the second pretreatment step was expected to yield a highly accessible material, it resulted in poor overall yields of sugar and ethanol. Problems in the reimpregnation with SO_2 were observed. Only small amounts were absorbed although 3% SO_2 was added. A probable explanation is that H_2SO_4 remained in the material after the first step, which prevented SO_2 absorption.

The mean value of absorbed SO_2 in all experiments was 1.64% of the water content, which is about half the amount added. However, variations in the amount absorbed between 0.83 and 2.33% were observed. This can affect the success of the pretreatment to a considerable extent, since the catalytic effect could be deficient owing to the low amount of SO_2 absorbed.

The total yield of mannose and glucose in the second pretreatment step—expressed as the sum of monomers in the liquid, and cellulose and hemicellulose in the solid—varied from 46 to 70 g/100 g of the solid material from the first pretreatment step. This corresponds to a yield of 73–110% based on the theoretical amount in the solid material after the first pretreatment step. The yield was highest for low-severity conditions and decreased with increasing severity in the second pretreatment step. It was assumed that the lignin is not degraded during the steam pretreatment when calculating the yields after the second pretreatment step. This assumption was made in determining the amount of carbohydrates in the solid material after the second pretreatment step.

Most of the remaining mannan from the first step was degraded during the second pretreatment step and obtained as mannose. The amount of glucan that was hydrolyzed and recovered in the liquid as glucose varied between 1 and 25% of the theoretical amount of glucan in the solid material from the first step. The amount of glucan that was hydrolyzed to glucose in the second pretreatment step reached a maximum for a severity factor of Log *Ro* = 4.23 (220°C, 5 min) (Fig. 2).

At low severity the recovery of material, taking into account glucan, mannan, their monomers, byproducts, and lignin, was close to 100%. However, for material pretreated at high severity the recovery was lower: for the highest severity not more than 66%. Handling losses cannot justify these poor mass balances. Some material is lost in the vapor, which was not

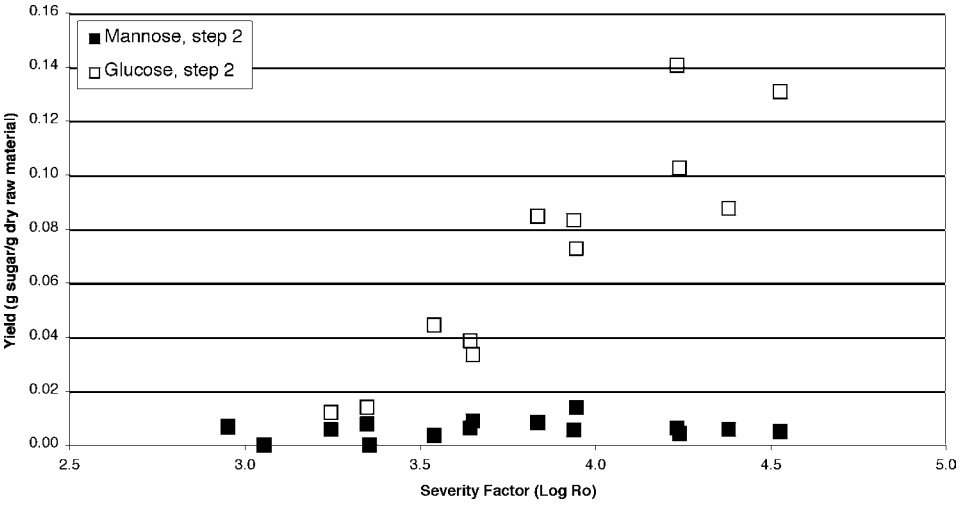

Fig. 2. Yield of monomeric glucose and mannose in liquid after second pretreatment step as function of severity of that step.

collected. Further studies in which this stream is considered are thus necessary. Losses arising from handling were determined by thoroughly washing the equipment with water and measuring the amount of solid material not recovered in the pretreated slurry. The average loss of solid material in the second pretreatment step was estimated to be 2.4% of the original dry material by weight.

The liquid after the second pretreatment step contained several byproducts. At low severity the concentrations of acetic acid, HMF, and furfural were very low, <0.5 g/L. The HMF concentration reached a maximum of 3 g/L for pretreatment at high severity. The furfural concentration never exceeded 0.75 g/L, which was expected, since almost all the pentoses were recovered as monomeric sugars in the liquid from the first pretreatment step (Fig. 3). Several other substances were detected as unidentified peaks in the chromatograms but were not quantified. These substances might be derived from the degradation of sugar and lignin.

All fermentation experiments on the liquid derived from the second pretreatment step showed good fermentability and no apparent inhibitory effects. The productivity for the first 4 h was 6 g of ethanol/(L·h), which was the same as in the reference solution.

Washed Material

Excessive washing of the material from the first step with water prior to reimpregnation resulted in a higher uptake (the desired 3%) of SO_2. However, SSF and enzymatic hydrolysis of this material did not result in an increase in overall yields compared with the moderately washed material exhibiting less SO_2 absorption.

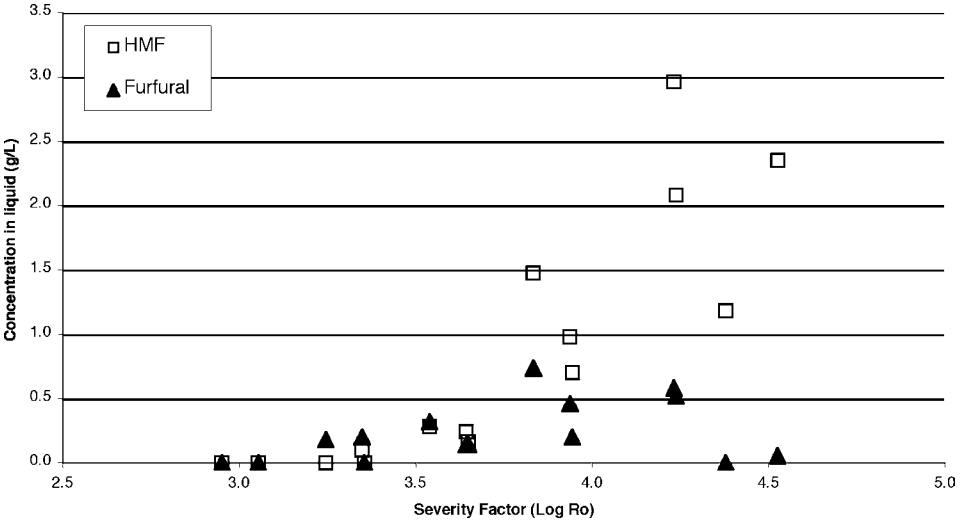

Fig. 3. Concentration of potential inhibitors in liquid after second pretreatment step as function of severity factor of pretreatment.

Enzymatic Hydrolysis

For enzymatic hydrolysis to be successful, the cellulose fibers must be accessible to the enzymes. More severe pretreatment results in a material that is more accessible to enzymatic attack. If the material is treated under very severe conditions, much of the cellulose will be hydrolyzed during the second pretreatment step without the use of enzymes. When treated at very severe conditions, the sugars are degraded during pretreatment to form inhibiting substances, which also cause a loss of substrate.

The sugar yields during the enzymatic hydrolysis step ranged from 12 to 20 g of sugar/100 g of dry raw material, depending on the pretreatment conditions. Figure 4 shows the glucose yield in the various steps. The highest yield in the enzymatic hydrolysis step, 19 g of glucose/100 g of dry raw material, was obtained for a severity of Log *Ro* = 3.94, corresponding to pretreatment conditions of 200°C and 10 min. For pretreatment at a severity above 4 the amount of glucan that was hydrolyzed in the enzymatic hydrolysis was smaller. This is an effect of the higher hydrolysis yield and increased degradation during the second pretreatment step, which leaves less material for enzymatic hydrolysis.

Simultaneous Saccharification and Fermentation

The success of SSF is dependent on the hydrolysis of the cellulose as well as on the fermentation of sugar to ethanol. A material pretreated at low severity in the second pretreatment step will not yield cellulose fibers that are accessible to enzymatic attack. However, if the material is treated

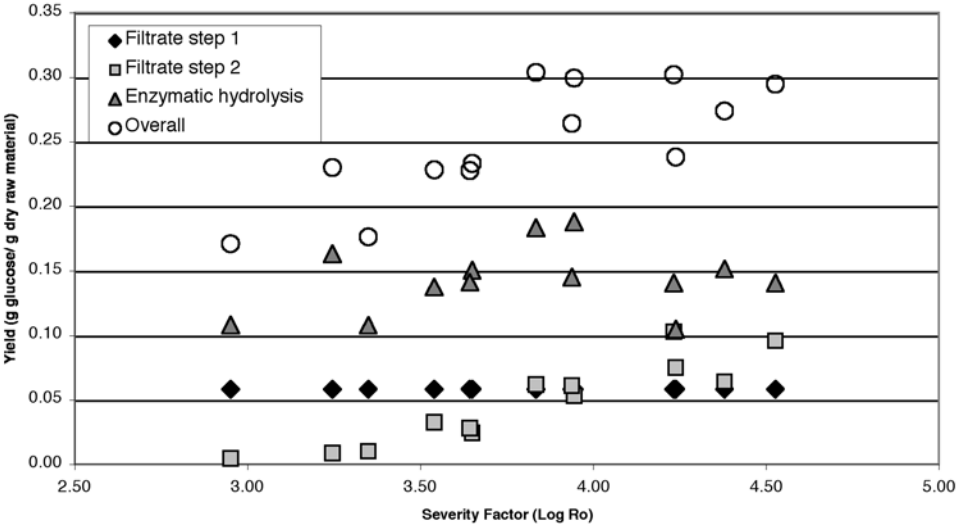

Fig. 4. Yield of glucose formed in each step as function of severity factor of pretreatment.

at high severity, inhibitors may form, which affect the fermentation and inhibit the yeast.

The yield of ethanol in SSF of the slurry from the second pretreatment step was calculated assuming that no lignin degradation occurred in the pretreatment. The highest yield of ethanol reached in SSF was 71% and was obtained following pretreatment at 230°C for 5 min. However, the highest overall ethanol yield (i.e., including both pretreatment steps and SSF) was 66%.

Overall Yields

The formation of glucose and mannose occurred in different steps of the process. Mannose was mainly formed during the first pretreatment step, with a yield of 88% of the theoretical value. In the second step, 2–8% of the theoretical amount of mannan was obtained, depending on the pretreatment conditions. Thus, the total yield of mannose was 90–96% of the theoretical.

Glucose was mainly obtained in the second pretreatment step and during enzymatic hydrolysis. A maximum of 21% of the theoretical amount of glucose was obtained in the second pretreatment step for pretreatment conditions of 220°C and 5 min. Considering enzymatic hydrolysis, the highest yield of glucose was 37%, obtained after pretreatment at 200°C for 10 min. However, the maximum yield following the second pretreatment step and enzymatic hydrolysis only reached 49%. In this case, pretreatment was performed at 220°C for 2 min.

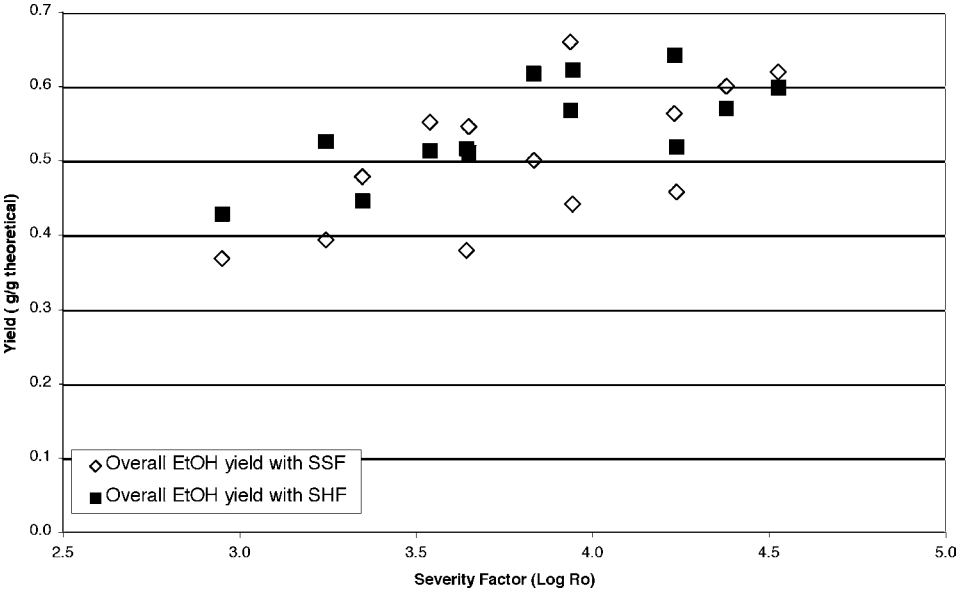

Fig. 5. Overall yields of ethanol following SSF and SHF as function of severity factor of pretreatment. In SHF the fermentation yield after enzymatic hydrolysis is assumed to be 90%.

The overall yield of fermentable sugars (i.e., following the two pretreatment steps and the enzymatic hydrolysis step) was about 70% for a range of pretreatment conditions with a severity factor of about 4 (*see* Fig. 4). The maximum yield of fermentable sugars (0.46 g/g dry raw material or 71%) was obtained following second-step pretreatment conditions of 220°C and 5 min.

The maximum yield of sugars obtained in our study is lower than in previous studies in which either two-step steam pretreatment with SO_2 impregnation in both steps (80%) *(9)* or H_2SO_4 impregnation in both steps (77%) *(10)* was utilized. The same raw material and evaluation methods were used as in the present study. Nguyen et al. *(6)* obtained an overall sugar yield of 82% using two-step steam pretreatment followed by enzymatic hydrolysis. However, the cellulase activity used in the present study was very much lower, 15 FPU/g of DM (25 FPU/g of cellulose), compared with the 60 FPU/g of cellulose used by Nguyen et al. *(6)*.

Figure 5 shows a comparison of SSF and SHF, in which a yield of 90% in the fermentation after enzymatic hydrolysis was assumed, which was the yield reached in the fermentation tests. When the material was steam pretreated in two steps followed by either SHF or SSF approximately the same ethanol yield resulted. The highest overall ethanol yield using SSF was 66%, reached at a severity factor of Log *Ro* = 3.94 (210°C, 5 min). For SHF the highest overall ethanol yield was 67% and was obtained for a severity factor of Log *Ro* = 4.23 (220°C, 5 min).

Previous results from one-step steam pretreatment have shown that SSF resulted in higher ethanol yields (67%) than SHF (60%) *(8,15)*. The use of antibiotics in SSF when evaluating the two-step steam pretreatment may cause a decrease in ethanol yield *(9)*. This may, to some extent, explain why the SSF configuration was inferior to the SHF configuration in this case, while the opposite was found when employing one-step steam pretreatment.

Two-step steam pretreatment with H_2SO_4 impregnation in both steps resulted in an overall ethanol yield of 65% with the SSF configuration and 69% with the SHF configuration *(10)*. The main reason for the lower yield following SHF in the present study, compared with the one using H_2SO_4 for impregnation in both steps, is a lower sugar yield in the second pretreatment step. When SO_2 was used for impregnation in both steps, the overall ethanol yield reached 69% with the SSF configuration and 72% with SHF. In this case, the higher sugar yield was mainly owing to a higher yield and higher sugar production in the enzymatic hydrolysis step.

Conclusion

Two-step steam pretreatment of softwood with impregnation by dilute H_2SO_4 in the first step and SO_2 in the second step resulted in lower sugar yields after enzymatic hydrolysis than did procedures incorporating impregnation with either SO_2 or H_2SO_4 in both steps. The ethanol yield after SSF was about the same as when H_2SO_4 was used in both steps, however, when SO_2 was used in both steps, the yield was higher. The highest overall ethanol yield reached with the SSF configuration was 66% of the theoretical (210°C, 5 min), whereas the highest overall sugar yield with the SHF configuration was 71% (220°C, 5 min).

This is contrary to what was expected based on previous results from one-step pretreatment with either H_2SO_4 or SO_2 *(11)*. Judging from those results, a first step using H_2SO_4 followed by a second step with SO_2 was thought to be superior. However, the results from our study show that it is not possible to predict the optimal conditions for two-step steam pretreatment based on a one-step pretreatment procedure. The combination of H_2SO_4 in the first step followed by SO_2 in the second step is thus not a better alternative than utilization of H_2SO_4 or SO_2 in both steps.

Acknowledgments

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