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Two-Step Steam Pretreatment of Softwood with SO_2 Impregnation for Ethanol Production

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Abstract

Two-step steam pretreatment of softwood was investigated with the aim of improving the enzymatic digestibility for ethanol production. In the first step, softwood was impregnated with SO_2 and steam pretreated at different severities. The first step was performed at low severity to hydrolyze the hemicellulose and release the sugars into the solution. The combination of time and temperature that yielded the highest amount of hemicellulosic sugars in the solution was determined. In the second step, the washed solid material from the optimized first step was impregnated once more with SO_2 and steam pretreated under more severe conditions to enhance the enzymatic digestibility. The investigated temperature range was between 180 and 220°C, and the residence times were 2, 5 and 10 min. The effectiveness of pretreatment was assessed by both enzymatic hydrolysis of the solids and simultaneous saccharification and fermentation (SSF) of the whole slurry after the second pretreatment step, in the presence of antibiotics. For each pretreatment combination, the liquid fraction was fermented to determine any inhibiting effects. At low severity in the second pretreatment step, a high conversion of cellulose was obtained in the enzymatic hydrolysis step, and at a high severity a high conversion of cellulose was obtained in the second pretreatment step. This resulted in an overall yield of sugars that was nearly constant over a wide range of severity. Compared with the one-step steam pretreatment, the two-step steam pretreatment resulted in a higher yield of sugar and in a slightly higher yield of ethanol. The overall sugar yield, when assessed by enzymatic hydrolysis, reached 80%. In the SSF configuration, an overall ethanol yield of 69% was attained.

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Index Entries: Enzymatic hydrolysis; spruce; simultaneous saccharification and fermentation.

Introduction

Ethanol fuel, which can be produced from various cellulosic materials, is proposed to be an alternative to gasoline. It can be manufactured from a whole array of natural materials containing cellulose or starch. The choice of substrate can thus be based on local conditions.

Softwood is an abundant feedstock in Sweden and can be used to produce fuel ethanol through, e.g., enzymatic hydrolysis and fermentation *(1–4)*. Softwood mainly comprises of three polymers: natural cellulose, a crystalline polymer that is associated in a matrix with two other polymers; lignin; and hemicellulose. This results in a material that is extremely resistant to enzymatic attack. To hydrolyze the cellulose by enzymes efficiently, pretreatment is necessary *(5)*. High yields can be achieved using enzymes, and this is therefore an attractive approach to the process *(4)*.

However, before fuel ethanol can be introduced commercially, the production cost must be competitive with that of fossil fuels. The greatest costs in the conversion of biomass to ethanol are those of the raw material *(1)* and of the enzyme *(6)*. Hence, it is important to utilize all the carbohydrate components present in the wood to make the process cost-effective *(6)*. The most important parameter in evaluating the production cost of ethanol has been found to be the yield *(7)*. A high recovery of the hemicellulosic sugars provides a higher ethanol yield and savings in capital cost *(8)*.

Steam pretreatment of softwood with SO_2 impregnation is an effective way to hydrolyze hemicellulosic sugars and to soften the structure of cellulose to enhance enzymatic accessibility *(6, 9–10)*. Prior to steam pretreatment, the material is impregnated with SO_2. The SO_2 impregnation of softwood, up to a concentration of 3%, increases the enzymatic accessibility, while higher concentrations have not shown any beneficial effects *(8,11)*.

Steam pretreatment can be evaluated with the severity correlation *(12)*, which describes the severity of the pretreatment as a function of treatment time (minutes) and temperature (°C), where T_{ref} = 100°C.

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

The utilization of the severity factor is an approximate method of evaluation since it assumes that a first-order reaction is taking place. This is, however, not the case in steam pretreatment. During steam pretreatment, the pentoses and hexoses formed from the hydrolyzed hemicellulose and cellulose may be further degraded to furfural, 5-hydroxymethylfurfural (5-HMF), levullinic acid and formic acid. Four major groups of inhibitors are present after steam pretreatment: organic acids, such as acetic acid, which is released from the hemicellulose on hydrolysis; lignin degradation

products; sugar degradation products; and extractives, which are all solubilized during pretreatment *(13)*. Sugar degradation products, such as furfural and 5-HMF, as well as phenolics from lignin degradation, can cause inhibition in the fermentation stage.

It is well known that more severe conditions during steam pretreatment will cause greater degradation of hemicellulosic sugars *(1,6,8,14–15)*. However, high severity is required to enhance the enzymatic digestibility of the cellulose fibers, especially for softwood *(16)*. When pretreatment is performed at high severity, the resulting material is easily digested by enzymes, but the hemicellulosic sugars are degraded to byproducts, as previously mentioned, which decreases the overall yield. On the other hand, if low severity is used, the recovery of hemicellulosic sugars is high, but the enzymes will not be able to digest the cellulose easily, which will also result in a poor overall yield. The formation of degradation products reduces the yield during the pretreatment step. There is also a risk of inhibition in the subsequent downstream process steps.

Because it is important to maximize the total sugar yield in the process, it is desirable to have high yields of both glucose and hemicellulosic sugars. In the present study, we focused on hexoses, since *Saccharomyces cerevisiae*, the yeast used, cannot ferment pentoses. However, from previous investigations, it is known that the maximum mannose and glucose yields are not obtained at the same degree of severity, because the hemicellulose is hydrolyzed at a lower degree of severity. Two-step steam pretreatment, with the first step performed at low severity to hydrolyze the hemicellulose and the second step in which the solid material from the first step is pretreated again, but at a higher degree of severity, can result in a higher overall sugar yield than a one-step steam pretreatment process. A two-step steam pretreatment process has been proposed in the literature *(2,10,11,14)*.

In the present study, a two-step steam pretreatment process was investigated in which the first step was optimized for the recovery of hemicellulose-derived fermentable sugars in the liquid. The solid material in the slurry was thoroughly washed with water and then pretreated in the second pretreatment step. The effect of pretreatment was assessed using both separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). The second pretreatment step was optimized with respect to the total ethanol yield after SSF and to the total yield of fermentable sugars after enzymatic hydrolysis for SHF.

Materials and Methods

Figure 1 illustrates the experimental procedure used. The softwood was impregnated with SO_2 and then steam pretreated. The resulting material was separated into a solid residue and a liquid. The liquid was analyzed for sugars and also fermented. The solid material was washed with water and then impregnated again with SO_2 and steam pretreated in the second pretreatment step. The resulting material was evaluated by SSF of the

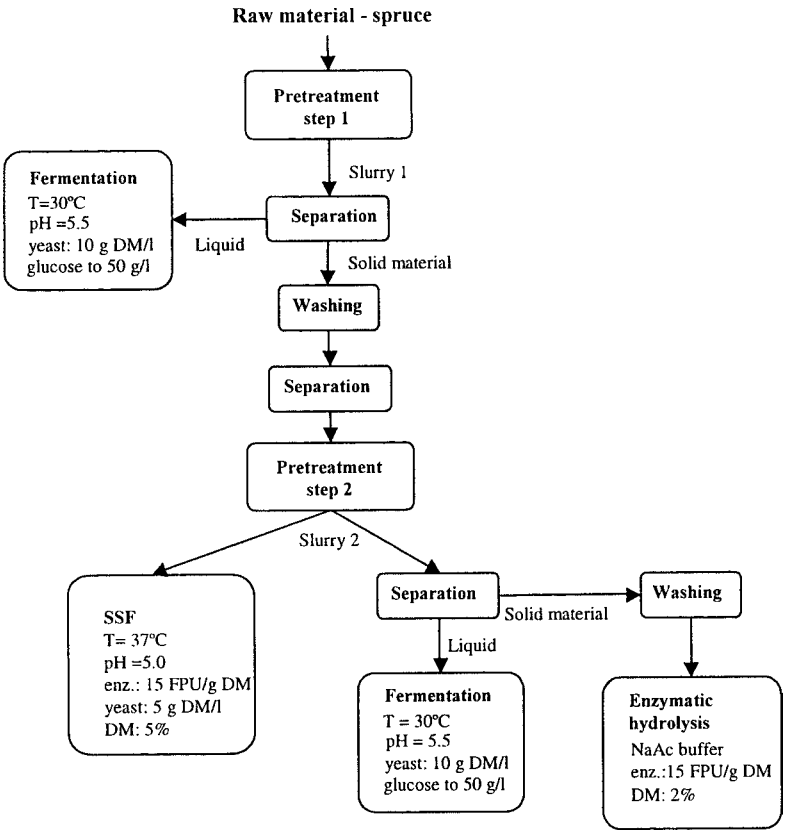

Fig. 1. Experimental setup for two-step steam pretreatment evaluation.

slurry, by enzymatic hydrolysis of the washed solid material and by fermentation of the liquid.

Raw Material

Fresh, chipped softwood, *Picea abies*, free from bark, was kindly provided by a sawmill (Harry Nilsson, Hästveda, Sweden). The wood chips were rechipped and fractionated further to a chip size between 2.2 and 10 mm. The composition was determined according to the Hägglund method *(17)* and is presented in Table 1. The material used for impregnation with SO_2 in the first step had a dry matter content of 44%. The material was stored in plastic bags at 4°C.

Pretreatment

Pretreatment was performed utilizing steam. The first pretreatment step was carried out in a 10-L reactor *(7)*, except where otherwise stated, while the second was performed in a 2-L reactor *(10)*. Several batches of pretreated material were produced in the 10-L reactor. After being well mixed, the material was used in the subsequent second pretreatment step.

Table 1
Composition of the Raw Materials
Used for the Evaluation of
First-Step (Material I) and Two-Step Pretreatment (Material II)

Composition	Dry matter material (I) (%)	Dry matter material (II) (%)
Glucan	46.5	46.5
Mannan	12.6	13.5
Lignin	27.8	27.9
Xylan	9.0	8.3
Galactan	3.9	1.7
Arabinan	1.1	1.2

First Pretreatment Step

The wood chips were impregnated with gaseous SO_2 (3% [w/w] based on the water content in the wood) in plastic bags for 20 min at room temperature and then steam pretreated. The optimization of the first pretreatment step was performed in the 2-L reactor at various temperatures (180, 190, 200, 210, and 220°C) and residence times (2, 5.5, and 10 min). The material was separated by filtration into a solid residue and a liquid. The liquid was analyzed for soluble sugars and degradation products. The composition of the solid material was determined using the Hägglund method *(17)*.

The temperature and residence time giving the highest yield of hydrolyzed hemicellulose were chosen. Several batches were performed under these conditions in the 10-L reactor and then mixed together. The pretreated material was separated by filtration into a solid residue and a liquid. The liquid was analyzed for soluble sugars and their degradation products. The solid material was washed thoroughly with water to remove all soluble substances, and the yield and composition of the solid material were determined *(17)*. The solid material was then used in the second pretreatment step. The conditions in the first pretreatment step were kept constant when varying the conditions in the second pretreatment step.

Second Pretreatment Step

The solid washed material was reimpregnated with 3% ([w/w], based on the water content) SO_2 in plastic bags for 20 min at room temperature. It was then steam pretreated in the 2-L reactor at various temperatures (180, 190, 200, 210, and 220°C) and residence times (2, 5, and 10 min). A portion of the pretreated material was used for SSF evaluation, and another portion was separated by filtration into a solid residue and a liquid for evaluation with SHF. The liquid was analyzed for soluble sugars and their degradation products. The amount of insoluble solids in the pretreated material was determined.

Determination of Oligosaccharides by Acid Hydrolysis

Acid hydrolysis of the liquid after the first pretreatment step was performed to determine the amount of oligomers. It was performed in two ways: by autohydrolysis using the acetic acid present in the liquid or by the addition of H_2SO_4. To a 2-mL sample of the liquid either 10.6 mL of H_2O and 1.4 mL of 1.0 mol/L H_2SO_4 or 12 mL of H_2O were added in 25-mL flasks. The flasks were autoclaved at 121°C for 4 h. After hydrolysis, $Ba(OH)_2$ was added to increase the pH. The neutralized liquid was filtered using 0.20-µm filters (MFS-13, Advantec MFS) before being analyzed for sugar content. Duplicate hydrolysis experiments were performed. During acid hydrolysis some sugar degradation may occur. This was not compensated for because it is difficult to determine accurately. However, the degradation is negligible according to the concentrations of 5-HMF and furfural obtained (data not shown) and will result in a slightly conservative estimation of the overall yield.

Enzymatic Hydrolysis

Enzymatic hydrolysis was used to assay the second steam pretreatment step. The enzymatic hydrolysis of the solid material from the second pretreatment step was performed using a commercial cellulase mixture, Celluclast 1.5L (65 filter paper units [FPU]/g and 17 β-glucosidase IU/g) supplemented with the β-glucosidase preparation Novozym 188 (376 β-glucosidase IU/g), both kindly donated by Novo Nordisk A/S (Bagsværd, Denmark). The filter paper activity was determined according to the procedure of Mandels et al. *(18)*, and β-glucosidase activity by the procedure of Berghem et al. *(19)*.

The enzymatic hydrolysis of the washed solid material was performed at 2% (w/w) dry matter to avoid endproduct inhibition in determination of the potential sugar yield. In the hydrolysis, 10 g of dry matter, 2.32 g of Celluclast and 0.52 g of Novozym were immersed in 0.1 mol/L of sodium acetate buffer (pH 4.8) to a total mass of 500 g, and under nonsterile conditions. The substrate was autoclaved (121°C for 20 min), but the enzyme solutions were not sterile. Hydrolysis was performed at 40°C for 96 h. Samples were withdrawn after 0, 2, 4, 6, 8, 24, 48, 72, and 96 h and analyzed for sugar content. All hydrolysis experiments were performed in duplicate.

Simultaneous Saccharification and Fermentation

SSF of the slurry from the second pretreatment step was used as an alternative method to assay the steam pretreatment. It was performed in 1-L fermentors (Belach AB, Stockholm, Sweden) using a total weight of 600 g, under nonsterile conditions. Nutrients were added to a final concentration of 0.5 g/L of $(NH_4)_2HPO_4$, 0.025 g/L of $MgSO_4 \cdot H_2O$ and 1 g/L of yeast extract. The substrate and nutrients were autoclaved separately (121°C for 20 min), but the enzyme solutions were not sterile. The slurry was diluted with water to obtain a final insoluble solids concentration of 5%

dry matter. Novozym 188 (1.56 g) and Cellulclast 1.5L (6.96 g) were used to give a final cellulase activity of 15 FPU/g of dry matter and a β-glucosidase activity of 23 IU/g of dry matter.

Compressed baker's yeast, *S. cerevisiae* (Jästbolaget AB, Rotebro, Sweden), was used at an initial concentration of 5 g of dry matter/L. The pH was initially adjusted with solid $Ca(OH)_2$ to 4.95–5.00 and was then maintained by the addition of 10% (w/w) NaOH. Antibiotics were added to prevent infection and formation of lactic acid. The concentration was 20,000 U/L of penicillin and 20 mg/L of streptomycin (Sigma-Aldrich, Irvine, UK). SSF was performed at 37°C for 72 h, and samples were withdrawn at 0, 2, 4, 6, 8, 24, 28, 32, 48, 52, 56, and 72 h and analyzed for ethanol, sugars, and byproducts. All the experiments were performed in duplicate.

Fermentation

Fermentation of the liquid was performed after the first and second pretreatment steps to investigate the fermentability and the extent of inhibition. The pH of the liquids was adjusted to 5.5 with 20% (w/w) $Ca(OH)_2$. Fermentation was performed in 25-mL glass flasks with a working volume of 20 mL consisting of 18.5 mL of the liquid, 0.5 mL of nutrients, and 1 mL of inoculum. The flasks were sealed with rubber stoppers through which hypodermic needles had been inserted for the removal of the CO_2 produced. The concentration of fermentable sugars (glucose and mannose) was adjusted by the addition of glucose to a total concentration of 50 g/L to obtain comparable fermentation results. The final concentration of nutrients was 0.5 g/L of $(NH_4)_2HPO_4$, 0.025 g/L of $MgSO_4 \cdot 7H_2O$, 0.1 mol/L of NaH_2PO_4, and 1 g/L of yeast extract. A reference solution was prepared with 30 g/L of glucose and 20 g/L of mannose, to serve as a control. *S. cerevisiae* was used at a concentration of 10 g of dry matter/L. The flasks were incubated at 30°C for 24 h and stirred with a magnetic stirrer. Samples were withdrawn at 0, 2, 4, 6, 8, and 24 h and analyzed for ethanol, sugars, and sugar degradation products. Fermentation 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, 5-HMF, and furfural were separated on an Aminex HPX-87H column (Bio-Rad) at 65°C using 5 mmol/L of 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

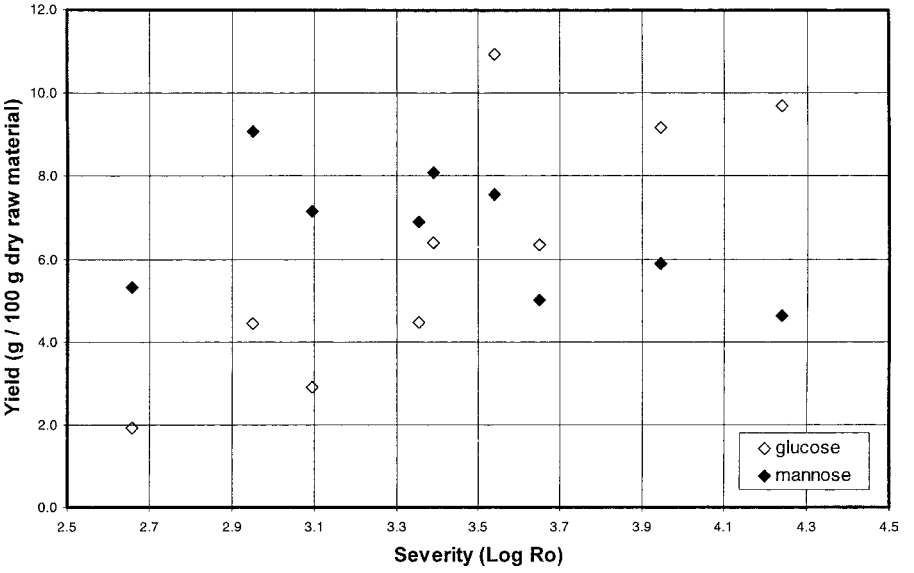

Fig. 2. Yield of glucose and mannose in liquid after first pretreatment step as function of severity of pretreatment.

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. The analysis of glucose in the liquid phase after pretreatment was also carried out on the HPX-87H column.

Results and Discussion

First Pretreatment Step

Overall yields of sugars and ethanol are based on the amount of glucose and mannose in the raw material. The first pretreatment step was optimized to obtain the maximum amount of mannose, the main hemicellulose sugar in softwood, in the liquid after pretreatment. A variety of pretreatment conditions, with severities varying between 2.66 and 4.24, were investigated.

The highest yields of mannose were obtained at severities of about 3 (Fig. 2). The pretreatment conditions that gave the highest yield of mannose, 9.1 g/100 g of raw material corresponding to 65% of the theoretical, were performed at 190°C and 2 min (Log *Ro* = 2.95). These were the conditions chosen for the first pretreatment step in the two-step pretreatment study and are in the range of severity that has been found by others. Wu et al. *(6)* found that the pretreatment conditions for the maximum recovery of soluble hemicellulosic sugars in the liquid as monomers and oligomers were 175°C, 7.5 min, and 4.5% SO_2, (Log*Ro* = 3.08).

Table 2
Recovery of Glucose and Mannose in
Liquid and Solid After First Pretreatment Step[a]

Sugar recovery (%) of theoretical yield	Present study	Ref. *2*	Ref. *6*	Ref. *15*
Glucose				
Total	97	—	—	—
Solid	88	—	—	—
Liquid	9	23	—	16
As oligomers (%)	6	5	21	12
As monomers (%)	94	95	79	88
Mannose				
Total	97	—	—	—
Solid	9	—	—	—
Liquid	88	63	—	87
As oligomers (%)	17	11	25	21
As monomers (%)	83	89	75	79

[a]Present study: 190°C, 2 min, 3% SO_2; ref. *2*: 212°C, 105 s, 0.35% H_2SO_4; ref. *6*: 170°C, 7.5 min, 4.5% SO_2; ref. *15*: 190°C, 3 min, 0.7% H_2SO_4.

When the first pretreatment step was performed on a larger scale (10-L reactor), the yield of mannose and glucose improved considerably compared with the yield obtained when the 2-L reactor was used. The total recovery of fermentable sugars, in the solid and the liquid as both monomers and oligomers, was 97% of the theoretical glucan yield and 97% of the theoretical mannan yield (Table 2). Ninety-one percent of the recovered mannan was obtained in the liquid, of which 17% was in oligomeric form and the rest (83%) as monomeric sugars. Ninety-one percent of the recovered glucan was obtained in the solid material and 9% was found in the liquid, of which 6% was present as oligomers. Autohydrolysis and acid hydrolysis with the addition of H_2SO_4 of the liquid after the first pretreatment step yielded the same results.

The yield was slightly higher than that obtained by Nguyen et al. *(15)* at 190°C, 3 min, and 0.7% H_2SO_4, in which the recovery of mannose in the liquid was 87% of the theoretical, of which 21% was found as oligomers. In another study by Nguyen et al. *(2)*, 90% solubilization of the hemicellulose was obtained at 212°C, 105 s and 0.35% H_2SO_4 (Log *Ro* = 3.54). However, only 63% of the mannose was present in the liquid, of which 11% was oligomers. In a study employing SO_2 impregnation (4.5%) and steam pretreatment at 175°C for 7.5 min, 77% of the hemicellulose was recovered as monomers in the liquid *(6)*. When autohydrolysis was performed on this liquid (no acid added), the yield of monomeric glucose increased by 27% and that of monomeric mannose by 33% *(20)*.

The concentration of sugars and other substances in the liquid depends on the amount of liquid obtained during pretreatment by the condensation

Table 3
Experimental Design of Second Pretreatment Step

Experiment	Temperature (°C)	Time (min)	Log *Ro*
1	180	5	3.05
2	180	10	3.36
3	190	2	2.95
4	190	5	3.35
5	190	10	3.65
6	200	2	3.25
7	200	5	3.64
8	200	10	3.94
9	210	2	3.54
10	210	5	3.94
11	210	10	4.24
12	220	2	3.83
13	220	5	4.23

of steam. This will, in turn, depend on the residence time and temperature used during pretreatment. The amount of byproducts is also important because it influences the yield while the concentration gives a better measure of the potential inhibition. The potential inhibitors determined in the liquid were acetic acid, 5-HMF, and furfural. 5-HMF and furfural were present at concentrations below 0.5 g/L, but acetic acid was only present in small amounts. The yield of ethanol in the fermentation of the liquid after pretreatment was 86% of theoretical, which was higher than that of the reference solution containing only glucose and mannose (80% of theoretical). This increase may be owing to the presence of organic acids including acetic acid and levulinic acid, which has been shown to increase the fermentation yield under certain conditions *(13)*. The productivity of ethanol after 4 h was about 50% of that of the reference solution but had recovered after 24 h.

Second Pretreatment Step

The second pretreatment step was performed using the washed solid material from the first pretreatment step, which was performed in the 10-L reactor at optimal conditions (190°C for 2 min). This material contained mainly glucan (57.9%) and lignin (36.9%) and small amounts of mannan (1.7%) and xylan (1.2%). All of the galactan and arabinan was hydrolyzed during the first pretreatment step, at least to a level that was measurable with the analysis method used. The investigation covered a severity range of 2.95–4.24 (Table 3). The second pretreatment step was evaluated using both SSF and enzymatic hydrolysis to determine the ethanol and glucose yield, respectively.

The total yield of glucose and mannose in the second pretreatment step—the yield in both the solid and liquid expressed as the sum of monomersand oligomers—varied between 45 and 67 g/100 g of solid

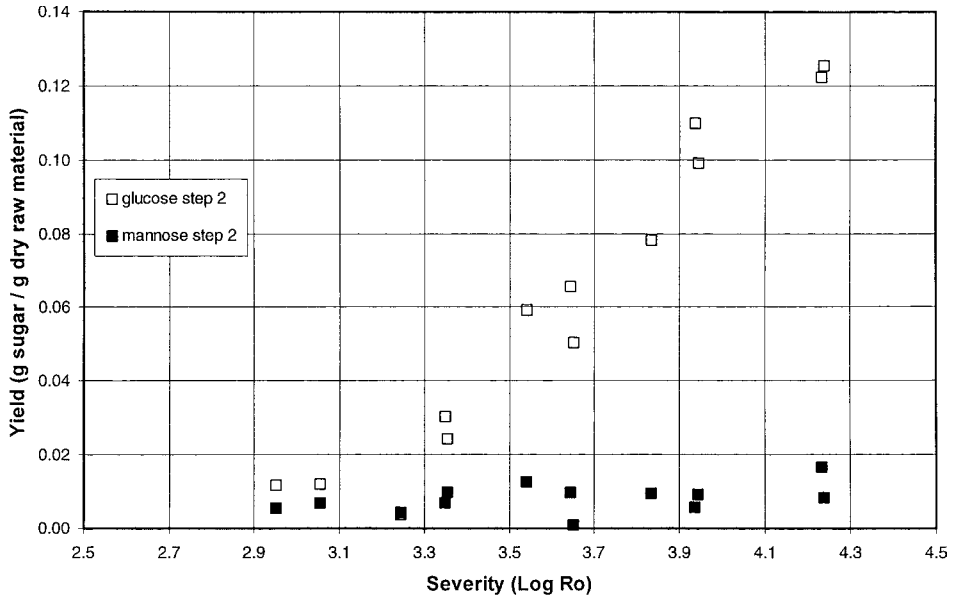

Fig. 3. Yield of glucose and mannose in liquid obtained in second pretreatment step as function of severity of that step.

material from the first step. This corresponds to a yield of 67–100% based on the theoretical amount in the solid material after the first pretreatment step. The yields during the second pretreatment steps are based on the assumption that the lignin is not degraded during steam pretreatment. This assumption is used to calculate the amount of carbohydrates in the solid material after the second pretreatment step. Most of the mannan, 0.6–1.9 g/100 g (32–100%) of the solid material from the first step, was obtained as monomeric sugar in the liquid. However, only a small portion of the glucan, 2–18 g/100 g (3–28%) was hydrolyzed and recovered as glucose monomers in the liquid (Fig. 3). The amount of hydrolyzed glucan and mannan increased with increasing severity in the pretreatment step. At low severity, the mass balance, taking into account glucan, mannan, their monomers, byproducts, and lignin, was close to 100% for pretreatment at low severity but decreased to 86% at the highest severity. Handling losses were determined by washing the pretreatment equipment thoroughly 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, 5-HMF, and furfural were very low, <0.5 g/L (Fig. 4). The 5-HMF concentration reached a maximum of about 2.7 g/L following pretreatment at the highest severity. The furfural concentrations never rose to concentrations above 0.5 g/L, which was expected since almost all the pentoses were recovered as monomeric sugars following the first pretreatment step.

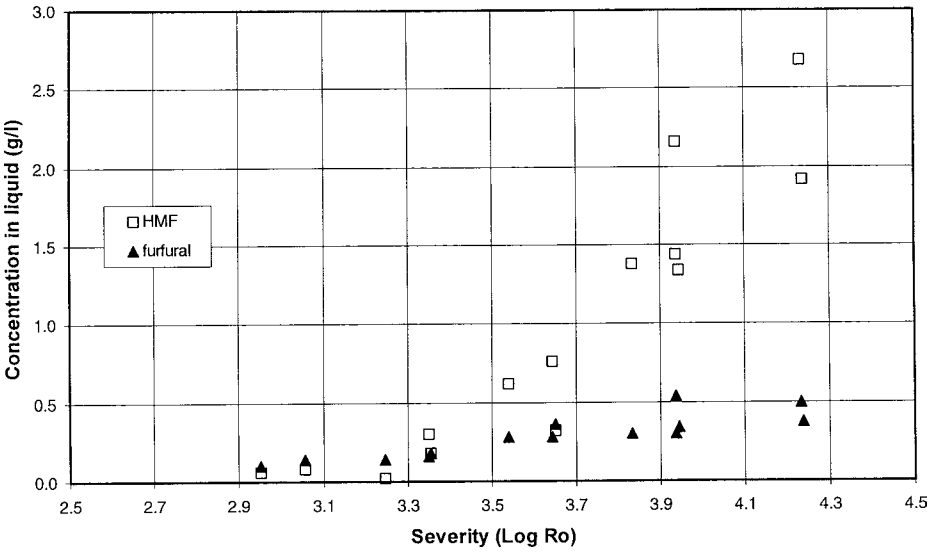

Fig. 4. Concentration of potential inhibitors in liquid obtained in second pretreatment step as function of severity of pretreatment.

Several other substances were detected as unidentified peaks in the chromatograms but were not quantified. These might be derived from the degradation of sugars or lignin. Fermentation of the liquids showed no apparent inhibitory effects for any of the pretreatment conditions. The ethanol yield was about 90% of the theoretical value, but always about 10% higher than the yield of the reference solution containing only glucose and mannose. The productivity was the same as for the reference solution. Acetic acid, which was present in all liquids, can improve the ethanol yield during fermentation, as mentioned previously *(13)*.

Enzymatic Hydrolysis

The solid material obtained after the second pretreatment step was washed and hydrolyzed enzymatically to assess the pretreatment. The yield was calculated assuming that no lignin was degraded during the pretreatment. The solids were assumed to consist only of lignin and cellulose. The sugar yields during the enzymatic hydrolysis step ranged from 47 to 105 g of glucose/100 g of the glucan in the material from the second pretreatment step, corresponding to 42–95% of the theoretical yields, depending on the preceding pretreatment conditions.

The success of the enzymatic hydrolysis depends on the accessibility of the cellulose fibers to the enzymes. A more severe pretreatment results in a material that is more accessible to enzymatic attack. However, if the material is treated under very severe conditions, most of the cellulose will be hydrolyzed in the second pretreatment step without the use of enzymes. At moderately severe pretreatment conditions, high conversion was obtained in the enzymatic hydrolysis, after 96 h, while at high severity a

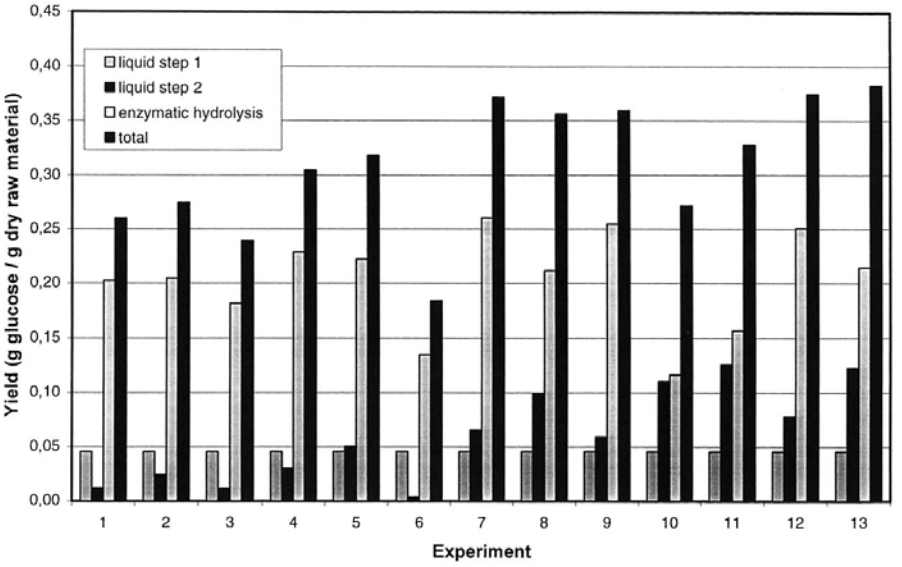

Fig. 5. Yield of glucose formed in each step for different pretreatment experiments. For details of experiments see Table 3.

high conversion of cellulose was obtained in the second pretreatment step. This resulted in a total yield of glucose that was almost constant over a broad severity range (Fig. 5). Evidently, pretreatment at the lower severities did not result in the optimal sugar yield.

The highest overall yields of fermentable sugars, i.e., including the two pretreatment steps as well as the enzymatic hydrolysis step, were obtained when the severity in the second pretreatment step was between 3.5 and 4.3. About 0.51 g of sugars (glucose and mannose) were formed per gram of dry raw material for several different pretreatment conditions. This corresponds to an overall yield of glucose and mannose of about 78% and was obtained for several temperatures ranging from 200 to 220°C. However, the maximum yield, 80%, was obtained for second-step pretreatment conditions of 220°C and 5 min.

Simultaneous Saccharification and Fermentation

Figure 6 shows the yield of ethanol in SSF of the slurry from the second pretreatment step. The yield of ethanol in the SSF step was calculated assuming that no lignin degradation occurred in the second pretreatment step. Several different pretreatment conditions yielded a total amount of ethanol of at least 0.27 L/kg of dry raw material, which corresponds to an ethanol yield of 65% of the theoretical, but in one case (190°C, 10 min) a yield as high as 69% was obtained.

Substrates pretreated at low severity, below 3.5, did not result in a high yield in SSF. The highest yields of ethanol during this step were obtained for experiments 5 and 10–12, attaining 80% of the theoretical amount in the

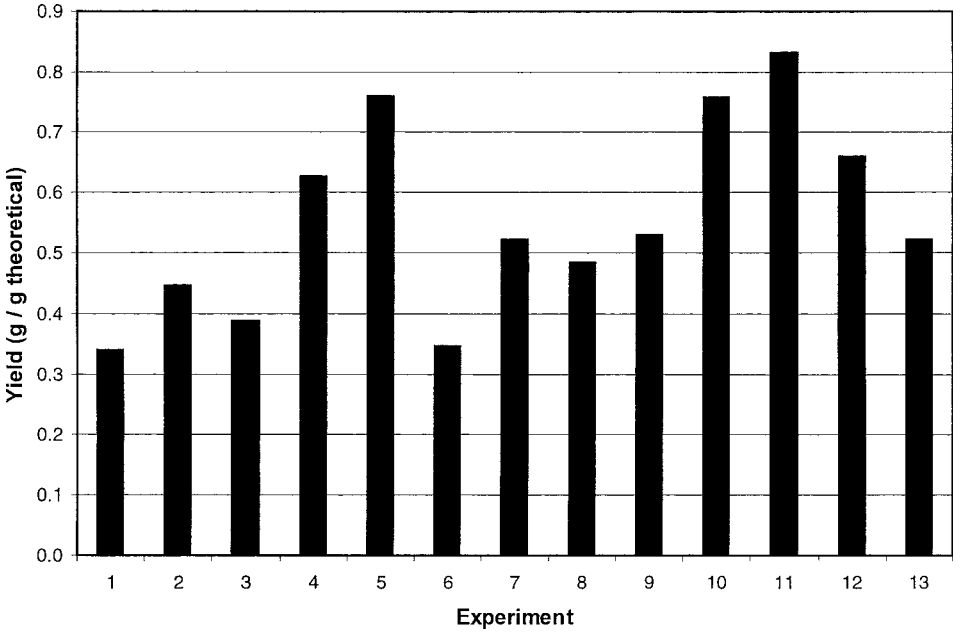

Fig. 6. Yield of ethanol in SSF for different conditions in second pretreatment step. See Table 3 for details of experiments.

SSF step. These experiments correspond to severities between 3.65 and 4.24. However, other experiments under different conditions but in the same severity interval did not result in such high yields. The yield in SSF seemed to be more affected by the temperature used than the actual severity of the pretreatment. Pretreatment at 220°C did not result in a favorable yield compared with the yield obtained following pretreatment at 210°C. These results show that the severity is an unreliable method of evaluation. Figure 7 shows a typical fermentation curve from SSF. In all cases, at least 80% of the final ethanol yield (after 74 h) was already reached after about 24 h.

The concentration of inhibitors (furfural, 5-HMF, and acetic acid) in the slurry increased with the severity of the second pretreatment step. Although present in the SSF, the ethanol yield was not affected by these potential inhibitors. The probable reason is that the concentrations of furfural and 5-HMF did not exceed 1 g/L and the concentration of acetic acid never reached 3.5 g/L in the SSF experiments.

Overall Yields

The formation of glucose and mannose, expressed as grams/grams of theoretical in the raw material, took place in different steps. Mannose was mainly obtained during the first pretreatment step with a yield of 88% of the theoretical. Oligomers constituted 20% of the liberated mannan fraction. In the second step, between 3 and 8% of the total theoretical amount

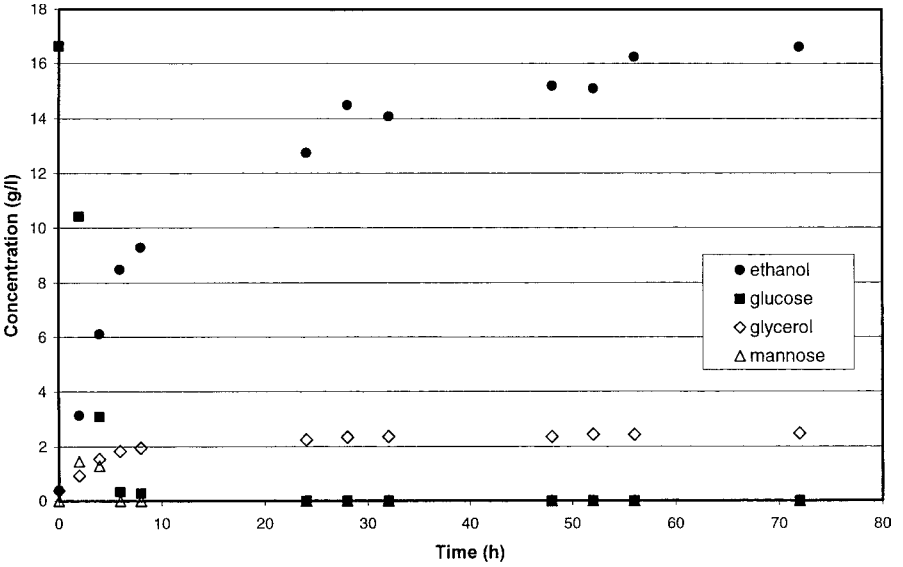

Fig. 7. Concentration of ethanol, glucose, glycerol, and mannose as function of time during SSF of material steam pretreated at 210°C for 5 min shown as mean values of duplicate runs.

of mannan was obtained, depending on the severity of pretreatment. No mannose was formed during enzymatic hydrolysis. The total yield of mannose reached 91–96%. Glucose was mainly obtained during the enzymatic hydrolysis, 31–52% of the theoretical total yield, after 96 h. The first pretreatment step yielded 9% of the theoretical amount of glucose in the liquid, including oligomers. The second pretreatment step yielded various amounts of glucose depending on the severity. The amounts obtained varied from only 2% for the lowest severity to about 25% of the theoretical total amount for the highest severity. The total amount of glucose produced using the SHF configuration was between 48 and 75% of the theoretical.

A comparison between SSF and SHF, with an assumed yield in the fermentation after the enzymatic hydrolysis of 90%, shows that for the material pretreated in two steps SHF gives a higher ethanol yield than does SSF (Fig. 8). This is in contrast to the one-step pretreatment in which SSF yielded much better results than SHF. One reason for this could be the addition of antibiotics in SSF to prevent random production of lactic acid and to give comparable results. Stenberg et al. *(21)* have shown that the use of antibiotics may cause a decrease in the yield in SSF.

Conclusion

In one-step steam pretreatment, the overall ethanol yield with SSF was 67% and the overall glucose yield using SHF was 66% *(10,22)*. This can be compared with the ethanol yield obtained in the two-step steam pretreatment process, which was about 69% with SSF and about 72% with SHF.

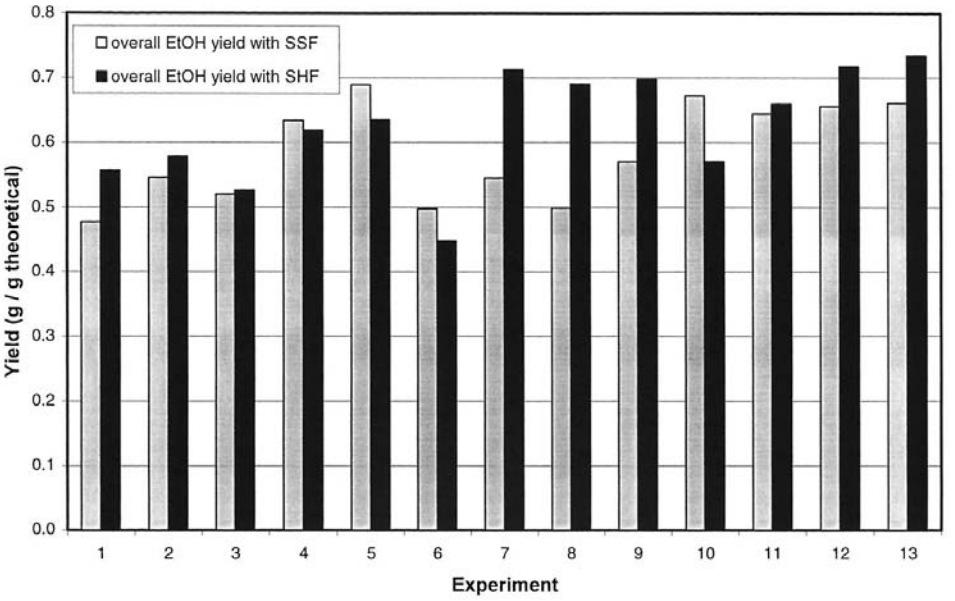

Fig. 8. Overall yields of ethanol in SSF and SHF for different pretreatment conditions. In SHF, the fermentation yield after enzymatic hydrolysis was assumed to be 90%.

These yields include fermentation of the liquid from the first pretreatment step with a yield of 90%. In the SHF case, fermentation of the liquid from the second pretreatment step and the hydrolysate after enzymatic hydrolysis were assumed with a yield of 90%, which was the average yield attained in the fermentation tests.

The total sugar yield in the SHF was 80%. This is slightly lower than the yield obtained by Ngyuen et al. *(16)* (82%) with two-step steam pretreatment followed by enzymatic hydrolysis. However, they used a cellulase activity of 60 FPU/g of cellulose in comparison with the 15 FPU/g of dry matter (≈25 FPU/g of cellulose) used in the present study.

The ethanol yield in the SSF step was better correlated with the pretreatment temperature than was the yield with the severity factor. The results show that evaluation of the pretreatment conditions using the severity factor is a less accurate method and that it should only be used for rough estimations.

The two-step steam pretreatment process has attractive advantages such as, higher ethanol yield and better utilization of the raw material, as well as lower consumption of enzymes. However, further evaluation is needed to determine whether these advantages outweigh the additional cost of adding another steam pretreatment step to the process.

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