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Two-step steam pretreatment of softwood by dilute H₂SO₄ impregnation for 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 by dilute H₂SO₄ impregnation to improve the overall sugar and ethanol yield has been investigated. The first pretreatment step was performed under conditions of low severity (180°C, 10 min, 0.5% H₂SO₄) to optimise the amount of hydrolysed hemicellulose. In the second step the washed solid material from the first pretreatment step was impregnated again with H₂SO₄ and pretreated under conditions of higher severity to hydrolyse a portion of the cellulose, and to make the cellulose more accessible to enzymatic attack. A wide range of conditions was used to determine the most favourable combination. The temperatures investigated were between 180°C and 220°C, the residence times were 2, 5 and 10 min and the concentrations of H₂SO₄ were 1% and 2%.

The effects of pretreatment were assessed by both enzymatic hydrolysis of the solids and with simultaneous saccharification and fermentation (SSF) of the whole slurry, after the second pretreatment step. For each set of pretreatment conditions the liquid fraction was fermented to determine any inhibiting effects. The ethanol yield using the SSF configuration reached 65% of the theoretical value while the sugar yield using the SHF configuration reached 77%. Maximum yields were obtained when the second pretreatment step was performed at 200°C for 2 min with 2% H₂SO₄. This form of two-step steam pretreatment is a promising method of increasing the overall yield in the wood-to-ethanol process.

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1. Introduction

During the past decades, global warming from the increased amount of greenhouse gases, mainly carbon dioxide, has become a major political and scientific issue. The main cause of global warming is believed to be the carbon dioxide formed by burning fossil fuels.

By using biofuels, the net emission of carbon dioxide to the atmosphere can be reduced. Ethanol, a biofuel, which can be produced from various cellulosic materials, has been proposed as an alternative fuel. It can be manufactured from numerous natural materials containing cellulose or starch.

Softwood is an abundant feedstock in Sweden and can be used to produce fuel ethanol through, for example, enzymatic hydrolysis and fermentation [1–4]. Softwood is mainly comprised of three polymers: natural cellulose, a crystalline polymer that is associated in a matrix with the two other polymers, lignin and

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hemicellulose. Because of the high lignin content, this material is very resistant to enzymatic attack. To improve the yield it is necessary to perform pretreatment prior to the enzymatic hydrolysis step.

The production cost must be competitive with that of fossil fuels for the commercial introduction of fuel ethanol. The highest costs in the conversion of biomass to ethanol are the cost of the raw material [1], and that of the enzymes. Consequently, it is very important to ensure a high degree of utilisation of all the carbohydrate components in the feedstock [5]. The overall yield has been found to be the most important parameter when evaluating the production cost of bioethanol [6].

Steam pretreatment of softwood by either H_2SO_4 or SO_2 impregnation constitutes an effective way of hydrolysing hemicellulose and softening the structure of cellulose to facilitate enzymatic attack [2,7,8]. Steam pretreatment can be evaluated with the severity correlation [9], which describes the severity of the pretreatment as a function of treatment time (minutes) and temperature ($^{\circ}\text{C}$), where $T_{\text{ref}} = 100^{\circ}\text{C}$.

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

When the pretreatment is performed under acidic conditions, the effect of pH can be taken into consideration by the combined severity [10] defined as

$$\text{Combined severity (CS)} = \text{Log}(Ro) - \text{pH}. \quad (2)$$

The pH can be calculated from the amount of sulphuric acid added to the material and the water content of the material. The utilisation of the severity factor and the combined severity factor for evaluation are approximate methods as they assume that a first-order reaction is taking place. However, this is not the case in steam pretreatment of wood.

During steam pretreatment, the pentoses and hexoses formed from the hydrolysed hemicellulose and cellulose may be further degraded to furfural, 5-hydroxymethylfurfural (HMF), levulinic acid and formic acid, together with other substances. Three major groups of potential inhibitors can be found in the liquid after dilute acid steam pretreatment: aliphatic acids, furan derivatives and phenolic compounds [11]. These compounds may cause inhibition in the fermentation step.

It is well known that more severe conditions during steam pretreatment will cause greater degradation of hemicellulosic sugars [1,5,12,13]. However, a high degree of severity is required to promote the enzymatic digestibility of the cellulose fibres, especially in softwood [7]. The formation of degradation products reduces the yield during the steam pretreatment step and the products may also cause inhibition in the following downstream process steps.

It is important to maximise the total sugar yield in the process and consequently it is desirable to have high yields of both glucose and hemicellulosic sugars. We have focused on hexoses, as they can be fermented by *Saccharomyces cerevisiae*, the yeast used in this study. Previous studies have shown that maximum hydrolysis of glucose and mannose is not obtained at the same pretreatment severity. Glucan demands pretreatment of higher severity than mannan to be completely hydrolysed. This suggests two-step steam pretreatment, with the first step performed at low severity to hydrolyse the hemicellulose and the second step, where the solid material from the first step is pretreated again, at higher severity. This approach can result in higher sugar yields than one-step steam pretreatment and has been proposed in the literature several times [2,7,12,14,15].

In the present study a two-step steam pretreatment process has been investigated. The conditions in the first pretreatment step were chosen to give a high 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 optimised with respect to the total ethanol yield after SSF and, for SHF, to the total yield of fermentable sugars after enzymatic hydrolysis.

2. Materials and methods

The experimental procedure employed in this study 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 analysed

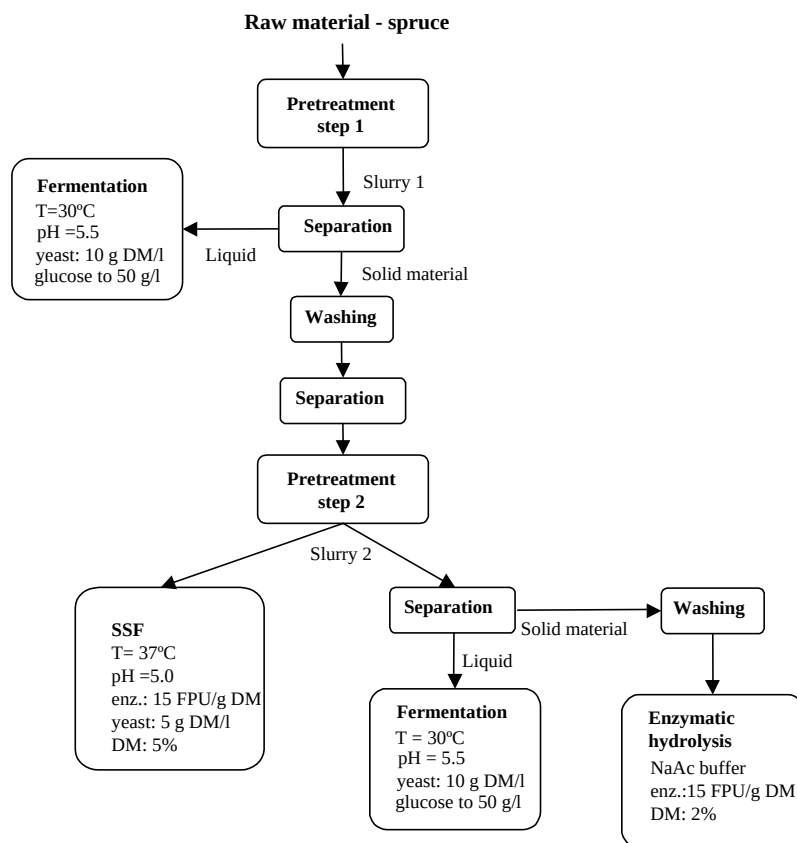


Fig. 1. The experimental set-up used for two-step steam pretreatment evaluation.

with regard to sugars and also fermented. The solid material was washed with water and then impregnated again with dilute H_2SO_4 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.

2.1. Raw material

Fresh softwood, *Picea abies*, free from bark, was used in this study. The sawdust was supplied by local sawmills. The composition was determined according to the Hågglund method [16] 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%.

Table 1

Composition of the raw material and the material after the first pretreatment step

Composition	Raw material (% of DM)	After 1st pretreatment step (% 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

2.2. Pretreatment

2.2.1. First pretreatment step

The first steam pretreatment step was optimised and performed at the Mid Sweden University in a 250-l

batch reactor located in Rundvik, Sweden [17]. 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 impregnated material had a DM content of 30%. The material was separated by centrifugation into a solid residue and a liquid. The liquid was analysed with regard to soluble sugars, and their degradation products. The composition of the solid material was determined with the Hågglund method [16]. The solid material was washed thoroughly with water to remove all soluble substances and the yield and composition of the solid material were determined [16].

2.2.2. Second pretreatment step

The second steam pretreatment step was performed at Lund University in a steam-explosion unit with a 2-l reactor [14]. The washed solid material, with a DM content of 37%, was re-impregnated with H_2SO_4 . Impregnation was performed with either 1% or 2% H_2SO_4 (w/w, based on the water content of the wood) in plastic bags overnight at room temperature. The impregnated material was steam pretreated in the second pretreatment step at various temperatures (180°C, 190°C, 200°C, 210°C, 220°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 analysed with respect to soluble sugars and their degradation products. The amount of insoluble solids in the pretreated material was determined.

2.3. 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; 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 H_2O and 1.4 ml, 1.0 mol l^{-1} H_2SO_4 or 12 ml H_2O were added in 25-ml flasks. The flasks were autoclaved at 121°C for 4 h. After hydrolysis, $\text{Ba}(\text{OH})_2$ was added to increase the pH and to precipitate sulphate ions. The neutralised liquid was

Table 2

Experimental design of the second pretreatment step

Experiment #	Temp. (°C)	Time (min)	% H_2SO_4	CS = Log Ro-pH
1	180	5	1	2.36
2	180	10	1	2.67
3	190	2	1	2.26
4	190	5	1	2.66
5	190	10	1	2.96
6	200	2	1	2.56
7	200	5	1	2.95
8	200	10	1	3.25
9	210	2	1	2.85
10	210	5	1	3.25
11	210	10	1	3.55
12	220	2	1	3.14
13	220	5	1	3.54
14	190	5	2	2.96
15	190	10	2	3.26
16	200	2	2	2.86
17	200	5	2	3.25
18	200	10	2	3.56
19	210	2	2	3.15
20	210	5	2	3.55
21	210	10	2	3.85
22	220	2	2	3.45
23	220	5	2	3.84

filtered using 0.20- μm filters (MFS-13, Advantec MFS, Inc., USA) before the sugar content was analysed. Duplicate hydrolysis experiments were performed. During acid hydrolysis some sugar degradation may occur. This was not compensated for, as it is difficult to determine accurately. However, the degradation is negligible judging from the concentrations of HMF and furfural obtained (data not shown) and will result in a slightly conservative estimate of the overall yield.

2.4. Enzymatic hydrolysis

Enzymatic hydrolysis was used to assay the second steam pretreatment step. This was performed using a commercial cellulase mixture, Celluclast 1.5 l (65 FPU g^{-1} and 17 β -glucosidase IU g^{-1}) supplemented with the β -glucosidase preparation Novozym 188 (376 β -glucosidase IU g^{-1}), both kindly donated by Novozymes (Bagsværd, Denmark). The filter paper activity was determined according to the

procedure of Mandels [18], and β -glucosidase activity by the procedure of Berghem [19].

Enzymatic hydrolysis of the washed solid material was performed at 2% (w/w) DM to avoid end-product inhibition in the determination of the potential sugar yield. In the hydrolysis, 10 g DM, 2.32 g Celluclast and 0.52 g Novozym were immersed in 0.1 mol l⁻¹ sodium acetate buffer (pH = 4.8) to a total mass of 500 g under non-sterile 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 analysed regarding the sugar content. All hydrolysis experiments were performed in duplicate.

2.5. SSF

SSF of the slurry from the second pretreatment step was used as an alternative method to assess the steam pretreatment conditions. This was performed in 1-l fermentors (Belach AB, Stockholm, Sweden) using a total weight of 600 g, under non-sterile conditions. Nutrients were added to a final concentration of 0.5 g l⁻¹ (NH₄)₂HPO₄, 0.025 g l⁻¹ MgSO₄ · H₂O and 1 g l⁻¹ yeast extract. The substrate and the 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% DM. 1.56 g Novozym 188 and 6.96 g Celluclast 1.5 l were used to give a final cellulase activity of 15 FPU g⁻¹ DM and a β -glucosidase activity of 23 IU g⁻¹ DM.

Compressed baker's yeast, *S. cerevisiae* (Jästbolaget AB, Rotebro, Sweden) was used at an initial concentration of 5 g DM l⁻¹. 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 the formation of lactic acid. The concentrations used were 20,000 U l⁻¹ of penicillin and 20 mg l⁻¹ of streptomycin, (Sigma-Aldrich Co. Ltd, 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 analysed regarding ethanol, sugars and by-products. All the experiments were performed in duplicate.

2.6. Fermentation

Fermentation of the liquid was performed after the first and the 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 nutrients and 1 ml inoculum. The flasks were sealed with rubber stoppers through which hypodermic needles had been inserted for the removal of the CO₂ produced. The concentrations of fermentable sugars (glucose and mannose) were adjusted by the addition of glucose to a total concentration of 50 g l⁻¹ to obtain comparable fermentation results. The final concentration of nutrients was 0.5 g l⁻¹ (NH₄)₂HPO₄, 0.025 g l⁻¹ MgSO₄ · 7H₂O, 0.1 mol l⁻¹ NaH₂PO₄ and 1 g l⁻¹ yeast extract. A reference solution prepared from 30 g l⁻¹ glucose and 20 g l⁻¹ mannose was also fermented. *S. cerevisiae* was used at a concentration of 10 g DM l⁻¹. 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 analysed with regard to ethanol, sugars and sugar degradation products. Fermentation experiments were performed in duplicate.

2.7. Analysis

The liquids after the pretreatment steps and all samples from the acid and the enzymatic hydrolysis, fermentation and SSF were analysed with HPLC (Shimadzu LC-10AT, Kyoto, Japan) with a refractive index detector (Shimadzu, Kyoto, Japan). Glucose, mannose, arabinose, galactose and xylose were separated using an Aminex HPX-87P column (Bio-Rad, Hercules, USA) at 80°C, using water as eluent, at a flow rate of 0.5 ml min⁻¹. Cellobiose, glucose, arabinose, lactic acid, glycerol, acetic acid, ethanol, HMF and furfural were separated on an Aminex HPX-87H column (Bio-Rad, Hercules, USA) at 65°C using 5 mmol l⁻¹ H₂SO₄ as the eluent, at a flow rate of 0.5 ml min⁻¹. All samples were filtered through a 0.20- μ m filter before HPLC analysis. Samples from the enzymatic hydrolysis and the liquid phases after the pretreatment steps were analysed on the HPX-87P column. However, because of interference between ethanol and mannose on that column, samples from

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

3. Results and discussion

3.1. 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 that could be used for ethanol production.

Ninety-three percent of the glucan was recovered after the first pretreatment step. Eighty-one percent was still present in the solid, whereas 12% was hydrolysed and present in the liquid as either oligomeric or monomeric sugars. Of the solubilised glucan, 87% was recovered as monomeric sugar (glucose) and the rest, 13%, was recovered as oligomeric sugar. The yield of mannan was even higher than that of glucan. One hundred percent of the mannan was recovered after the first pretreatment step. Twelve percent was still present in the solid and the remaining part, 88%, was solubilised and present in the liquid. The mannan present in the liquid consisted of 88% monomeric sugar (mannose) and 12% oligomeric sugars (Table 3). The yields of solubilised glucose and mannose as monomeric and oligomeric sugars were about the same as those obtained by Nguyen et al. at 190°C, 3 min and 0.7% H₂SO₄ and by Kim et al. at 185°C, 4 min and 0.66% H₂SO₄ [13,20]. However, Kim et al. [20] observed a higher glucose yield. The rather low recovery of glucose (93%) in the present study may be due to the use of a large pretreatment reactor (250 l) and separation unit. Material may be lost in the equipment when only one batch is treated. The missing 7% could not be accounted for by the degradation of sugars as HMF was present only in very small amounts. Autohydrolysis and acid hydrolysis with the addition of H₂SO₄ to the liquid after the first pretreatment step yielded the same results.

In the liquid, only small amounts of furfural and HMF were present, at concentrations of 0.7 and 1.4 g l⁻¹, respectively. Acetic acid was present at a concentration of 3.7 g l⁻¹. The total amounts of these substances were 0.6 g HMF per 100 g dry raw

material, 0.3 g furfural per 100 g dry raw material and 1.6 g acetic acid per 100 g dry raw material. The amount of acetic acid corresponds well with the degree of acetyl substitution in galactoglucomannan.

The concentrations of sugars and other substances in the liquid after pretreatment depend on the amount of liquid obtained during pretreatment by the condensation of steam. This will depend on the residence time and the temperature used during the process. However, not only the concentration of by-products is of importance, but also the yield based on the amount of raw material, as a large amount of these substances may lead to a lower ethanol yield. Kim et al. showed that pretreatment at 185°C for 4 min with 0.66% H₂SO₄ resulted in 2.5 g HMF per 100 g dry raw material [20], which is slightly more than that obtained in this study.

The yield after fermentation of the liquid from the first pretreatment step was 94% of the theoretical fermentation yield (data not shown), which was the same as for the reference solution. This indicates that no inhibition occurred, which was expected, as the possible inhibitors were present at very low concentrations, due to the low degree of severity used in the pretreatment. The productivity of ethanol after 4 h of fermentation was about half of that of the reference solution, but after 24 h about the same yield was reached for both reference solution and the liquid from the first pretreatment step.

3.2. 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%) arabinan (0.6%) and xylan (1.6%) (Table 1). The investigation covered a combined severity range of CS = 2.26–3.85 (Table 2). The second pretreatment step was evaluated using both SSF and enzymatic hydrolysis to determine the ethanol yield and the glucose yield, respectively.

The total yield of mannose and glucose in the second pretreatment step, expressed as the sum of monomers and oligomers in the liquid and polymers in the solid, varied between 20 and 68 g/100 g of the solid material from the first pretreatment step. This corresponds to a yield of 33–100% based on the

Table 3

Recovery of glucose and mannose in the liquid and solid after the first pretreatment step

Sugar recovery	(%) of theoretical yield	Present study	[2]	[13]	[20]
Glucose	Total	93	—	—	103
	Solid	81	—	—	91
	Liquid	12	23	16	12
	As oligomers (%)	13	5	12	9
	As monomers (%)	87	95	88	91
Mannose	Total	100	—	—	96
	Solid	12	—	—	10
	Liquid	88	63	87	86
	As oligomers (%)	12	11	21	14
	As monomers (%)	88	89	79	86

Present study—180°C, 10 min, 0.5% H₂SO₄. [2]—212°C, 105 s, 0.35% H₂SO₄. [13]—190°C, 3 min, 0.7% H₂SO₄. [20]—185°C, 4 min, 0.66% H₂SO₄.

theoretical amount in the solid material after the first pretreatment step. The yields during the second pretreatment step are based on the assumption that the lignin is not degraded during steam pretreatment. This assumption was employed to estimate the amount of carbohydrates in the solid material after the second pretreatment step.

During the pretreatment some carbohydrates may form pseudo-lignin causing the amount of available carbohydrates to be lower than assumed. On the other hand acid soluble lignin may be found in the liquid leaving the solid material with less lignin than predicted. This will influence the yields of the second pretreatment step and the enzymatic hydrolysis step, especially at high severity. However, the overall sugar yield and the yields calculated, as g per 100 g raw material for the individual steps, will not be affected.

Most of the mannan in the solid material after the first pretreatment step was obtained as monomeric sugar in the liquid after the second pretreatment step. The amount of glucan that was hydrolysed and recovered in the liquid as glucose varied between 14% and 77% of the theoretical (4–22 g per 100 g of the solid material from the first step) (Fig. 2). The amount of glucan hydrolysed to glucose in the second pretreatment step reached a maximum at a combined severity of CS = 3.1–3.2. At higher degrees of severity the glucose was further degraded to HMF and probably levulinic acid. Most of the remaining mannan from the first step was hydrolysed to mannose during the second pretreatment step. However, at high severity,

a low recovery of mannose was observed in the second step and mannose was probably degraded to HMF and levulinic acid.

At low severity, the mass balance, taking into account glucan, mannan, their monomers, by-products and lignin, was close to 100%. However, at high severity, less of the material could be accounted for after the pretreatment. For the highest degree of severity only 63% of the material was accounted for after the pretreatment. Handling losses cannot justify these losses of material. Other “losses” may be accounted for by by-products not analysed, gases, etc., and is a subject for further studies. Handling losses 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 many by-products. At low severity the concentrations of acetic acid, HMF and furfural were very low, less than 2 g l⁻¹ (Fig. 3). The HMF concentration reached a maximum of 3.9 g l⁻¹ following pretreatment at moderate severity. After pretreatment at higher severity the amount of HMF was lower. This is probably due to further degradation of HMF. The furfural concentration never exceeded 1.5 g l⁻¹, which was expected as almost all the pentoses were recovered as monomeric sugars in the liquid from the first pretreatment step. Several other substances were seen as unidentified peaks in the chromatograms but

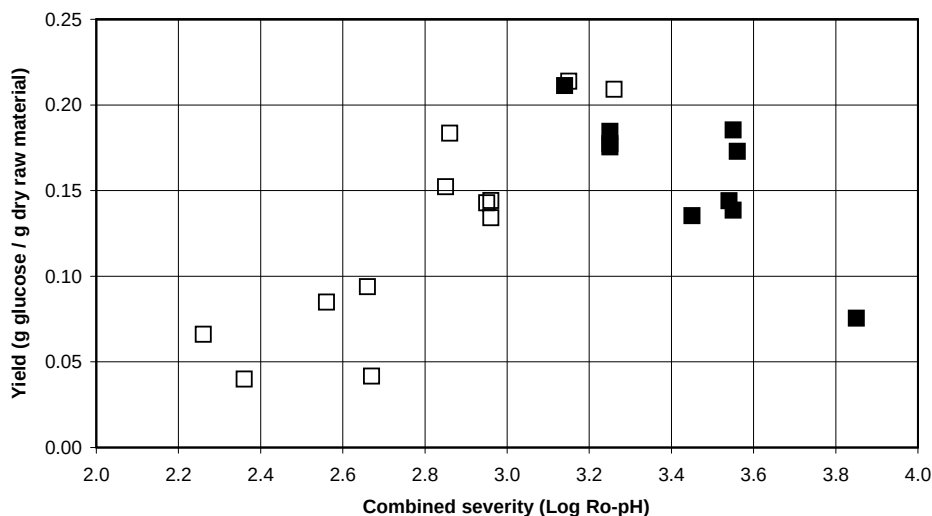


Fig. 2. The yield of monomeric glucose in the liquid after the second pretreatment step as a function of the combined severity. (□) Fermentable samples and (■) Non-fermentable samples.

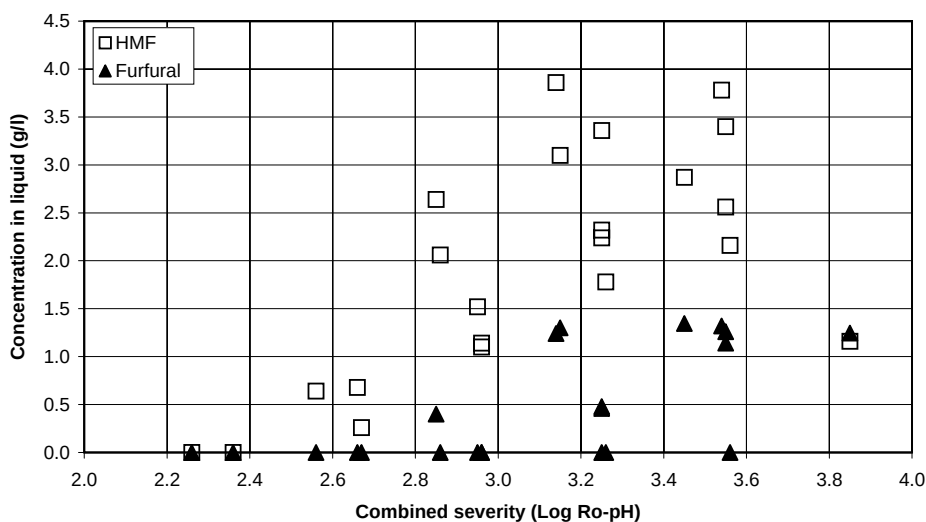


Fig. 3. Concentration of potential inhibitors in the liquid after the second pretreatment step as a function of the combined severity of the pretreatment.

not quantified. These substances are derived from the degradation of sugar and lignin. At least one unidentified peak made a major contribution and increased in size (amount) with the severity of the pretreatment and interfered with the acetic acid peak. This was probably levulinic acid. This assumption is supported

by the fact that the amount of HMF increased up to CS = 3.2 followed by a decrease and it is known that levulinic acid is obtained as a reaction product from the degradation of HMF.

Fermentation of the liquid derived from pretreatment at low severity showed good fermentability and

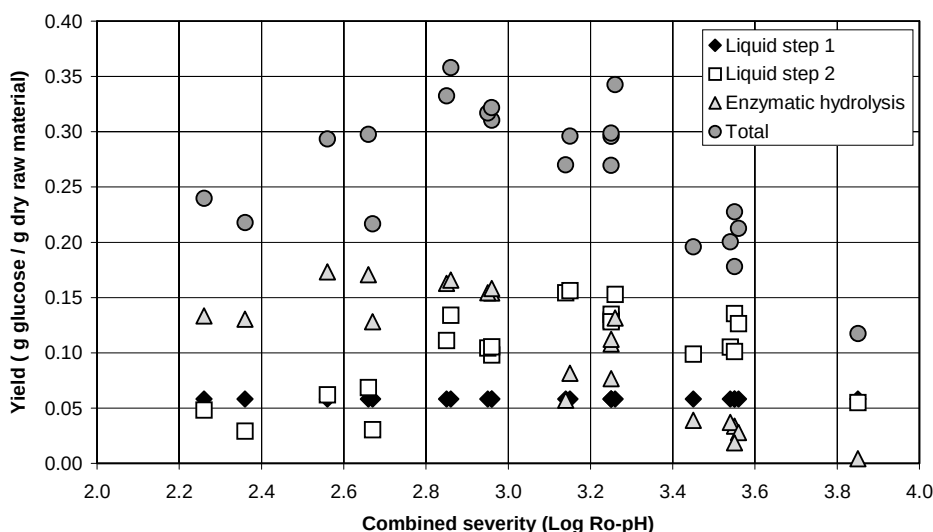


Fig. 4. The yield of glucose formed in each step as a function of the combined severity of the second pretreatment step.

no apparent inhibitory effects. However, when higher combined severity was used, (above $CS = 3.2$) the fermentation was poor (Fig. 2). The concentration of HMF, and other possibly inhibiting substances increased markedly at high severity. When good fermentability was obtained the final ethanol yield was as high as for the reference solution, i.e. 83–100% of the theoretical yield. The productivity during the first 4 h of the fermentation was about half of that of the reference solution, which was about 5 g ethanol l^{-1} h.

3.3. Enzymatic hydrolysis

For enzymatic hydrolysis to be successful the cellulose fibres must be accessible to the enzymes. More severe pretreatment results in a material that is more accessible to enzymatic attack. However, if the material is treated under very severe conditions much of the cellulose will be hydrolysed already during the second pretreatment step. When treated under very severe conditions sugar degradation during pretreatment causes a loss of substrate as well as undesirable production of inhibiting substances.

The solid material obtained after the second pretreatment step was washed and hydrolysed enzymatically to assess the effects of pretreatment. The yield was calculated assuming that no lignin was degraded during pretreatment. The solid material was assumed

to consist of lignin and cellulose only. As discussed earlier this assumption will not affect the overall yield, but may influence the yield of the enzymatic hydrolysis step. The sugar yields during the enzymatic hydrolysis step ranged from 6 to 99 g glucose per 100 g of the glucan in the material from the second pretreatment step, depending on the pretreatment conditions. No mannan was found in the material to be hydrolysed following the second pretreatment step.

Enzymatic hydrolysis gave the highest yields for pretreatment at a combined severity of $CS = 2.56$, corresponding to pretreatment conditions of 200°C, 2 min and 1% H_2SO_4 . This resulted in 17 g glucose per 100 g dry raw material (Fig. 4). Materials pretreated at a combined severity higher than 3.4 in the second pretreatment step resulted in very poor enzymatic hydrolysis, if any.

The highest overall yields of fermentable sugars from the two pretreatment steps, as well as the enzymatic hydrolysis step, were obtained when the combined severity in the second pretreatment step was around 2.8–3.0. The overall yield of glucose and mannose was about 75% and was obtained under several different pretreatment conditions with varying temperatures, residence times and H_2SO_4 concentrations. From 1 g of dry raw material 0.48 g of fermentable sugars were formed. The maximum yield of sugar, 77%, was obtained with second step

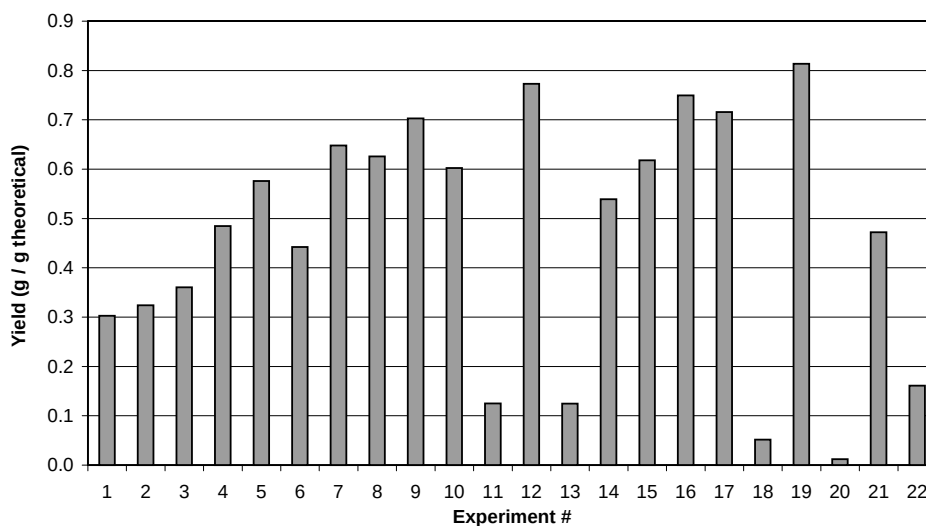


Fig. 5. The yield of ethanol in SSF for different conditions in the second pretreatment step. See Table 2 for details of experiments.

pretreatment conditions of 200°C, 2 min and a H₂SO₄ concentration of 2%.

The maximum yield of sugar obtained in this study (77%) is slightly lower than that obtained by Nguyen et al. (82%) using two-step steam pretreatment followed by enzymatic hydrolysis [7]. However, in our study a much lower cellulase activity was used; 15 FPU g⁻¹ DM (25 FPU g⁻¹ cellulose) compared with the 60 FPU g⁻¹ cellulose used by Nguyen et al. About the same maximum yield (80%) was obtained in a previous study on two-step steam pretreatment with SO₂ impregnation in both steps, using the same raw material and evaluation methods as in the present study [15].

3.4. SSF

The outcome of SSF depends on the hydrolysis of the cellulose as well as the fermentation of sugar to ethanol. A material pretreated at low severity in the second pretreatment step will result in cellulose fibres that are not very accessible to enzymatic attack. However, if the material is treated at high severity inhibitors may form, which affect the fermentation and inhibit the yeast.

The yield of ethanol after SSF of the slurry from the second pretreatment step was calculated assuming that no lignin degradation occurred in the

pretreatment. Yields after SSF reached as high as 80% of the theoretical (Fig. 5). However, the overall ethanol yield, i.e. including both pretreatment steps and SSF did not result in yields higher than 65%. The highest yield was obtained at a combined severity of CS = 2.86 (200°C, 2 min and a H₂SO₄ concentration of 2%), which is the same as for the evaluation with enzymatic hydrolysis.

The highest yields of ethanol during SSF were obtained for experiments 12, 16 and 19, corresponding to a combined severity between 2.86 and 3.15 (Table 2). However, several experiments in the same severity range, but under different pretreatment conditions, did not result in as high ethanol yields. These results indicate that the concept of the severity factor and the combined severity are unreliable methods for the evaluation of SSF. They may only be used for rough estimates. The ethanol yield in SSF is mainly affected by the concentration of H₂SO₄ and the temperature during the second pretreatment step.

3.5. Overall yields

The formation of glucose and mannose, expressed as g/g theoretical amount in the dry raw material, occurred in different steps of the process. Mannose was mainly formed during the first pretreatment step with

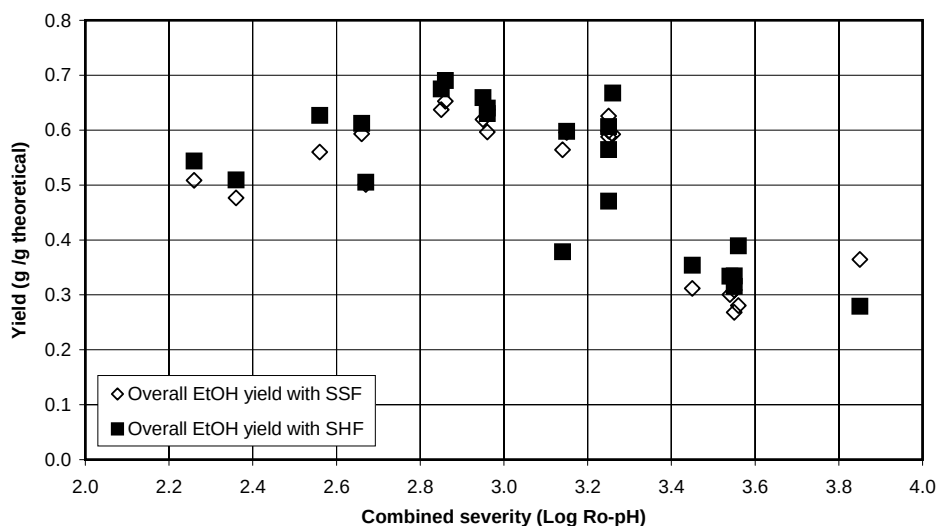


Fig. 6. The overall yields of ethanol in SSF and SHF as a function of the combined severity in the second pretreatment step. In SHF the fermentation yield after enzymatic hydrolysis was assumed to be 90%.

a yield of 88% of the theoretical amount. Oligomers constituted 12% of the liberated mannan fraction. In the second step 2–12% of the theoretical amount of mannan was obtained, depending on the pretreatment conditions. Thus, the total yield of mannose was 90–100% of the theoretical.

Glucose was mainly obtained in the second pretreatment step and during enzymatic hydrolysis. A maximum of 30% of the theoretical amount of glucose was obtained in the second pretreatment step and another 34% in the enzymatic hydrolysis. These maxima did not occur under the same pretreatment conditions; therefore, the maximum combined glucose yield was only 60%.

Fig. 6 shows a comparison between SSF and SHF, with an assumed yield from fermentation after the enzymatic hydrolysis of 90%, which was the yield obtained in the successful fermentation experiments. The material pretreated in two steps followed by SHF gave a higher ethanol yield than the SSF configuration. Previous results from one-step steam pretreatment showed that SSF gave higher ethanol yields. However, the two-step steam pretreatment with SO_2 impregnation also resulted in higher overall yields with SHF than SSF, [15].

Stenberg et al. have shown that, when using one-step steam pretreatment, the overall ethanol yield

with SSF was 67% while the overall hexose yield in SHF was 75%, [14,21]. In the present study two-step steam pretreatment with impregnation of H_2SO_4 in both steps resulted in an overall ethanol yield with SSF of 65% and an overall yield of glucose and mannose in SHF of 77%.

One reason for the lower yield in SSF than SHF when using two-step steam pretreatment could be the use of antibiotics in SSF to prevent random production of lactic acid and to give comparable results. The same conclusion, i.e. the SHF results in a higher overall yield than SSF, was drawn in a previous study of two-step steam pretreatment with SO_2 impregnation [15]. Stenberg et al. have shown that the use of antibiotics in SSF may cause a decrease in the ethanol yield [22].

4. Conclusions

The ethanol yield after two-step steam pretreatment followed by SSF reached 65% of the theoretical yield. However, when using SHF the yield was increased to 69%, when the fermentation yield after enzymatic hydrolysis was assumed to be 90%, which was the yield obtained in the fermentation experiments. The SHF configuration results in higher yields than the SSF configuration. This was not the case in one-step

steam pretreatment, where SSF showed the most promising results.

The severity factor and the combined severity are not accurate measures in the evaluation of steam pretreatment, and should only be used for rough estimates. The yield in SSF was better correlated with the temperature and the concentration of H_2SO_4 than with the combined severity.

The two-step steam pretreatment process with dilute H_2SO_4 impregnation shows attractive advantages, such as high ethanol yield, better utilisation of the raw material and lower consumption of enzymes. However, further evaluation is required to determine whether these advantages outweigh the disadvantages of adding another steam pretreatment step to the process.

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