0273-2289/05/125/0175/$30.00

Carbohydrate Reactions During High-Temperature Steam Treatment of Aspen Wood

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Received July 16, 2004; Revised February 2, 2005;
Accepted February 4, 2005

Abstract

Aspen wood was treated with steam at different time-temperature severity factors. Analysis of the amounts of acids released revealed a relationship between the acidity and the formation of furfural and hydroxymethyl furfural as degradation products from carbohydrates. It is suggested that two concurrent or consecutive mechanisms are responsible for the observed results: a homolytic cleavage and an acid hydrolysis of glucosidic linkages in the polysaccharides. By preimpregnating the wood with alkali, hydrolysis can be eliminated, resulting in a much cleaner depolymerization of the polysaccharides without any further acid-catalyzed degradation. The enzymatic digestibility of the steam-treated wood material for the formation of glucose was compared with that of steam-exploded wood. A more efficient route for glucose production from steam-exploded wood was found as long as the biomass-pretreated material was homogeneous and without shives.

Index Entries: Aspen wood; steam treatment; steam explosion; depolymerization; acid-catalyzed degradation; furfural; hydroxymethyl furfural; enzymatic hydrolysis; acid hydrolysis.

Introduction

The exploitation of biomass for the production of energy, food, fiber, and chemicals has increased in importance owing not only to environmental concerns but also to the anticipated future shortage of petroleum. Until now, most of the work has been focused on the polysaccharides because these constitute the predominant constituent. For example, aspen, one of

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the fastest-growing wood species, contains about 77% carbohydrates. Of this amount, approx 65% is cellulose *(1)*. In addition to research focused on the production of pulp from aspen wood *(2–4)*, work has been directed toward making pure cellulose *(1,5)* as well as low molecular weight chemicals after the depolymerization and fermentation of sugars *(6)*. When acidic conditions are encountered in the first separation step, however, as in steam treatment, a substantial loss of carbohydrates takes place, and the yields of valuable products are comparatively low.

The steam explosion process is one of the most attractive for the initial fractionation of the biomass material into its components, cellulose, hemicellulose, and lignin *(7)*. In this process, the biomass is treated with pure steam at a high temperature, usually 200–220°C, for a few minutes, followed by a sudden decompression, which forces the fibrous material to "explode" into separated fibers and fiber bundles. When no other chemicals are added to the process, the high-temperature steam leads to the release of acids from the acetylated wood components, and these, in turn, catalyze hydrolytic reactions in the wood polymers. This autohydrolytic mechanism has been linked to changes occurring in the lignin structure in aspen wood *(8–11)*. It has also been reported that there is a substantial loss of both cellulose and hemicelluloses during steam explosion although the acidity is low *(1,5,12)*, and both hydroxymethyl furfural (HMF) and furfural have been identified as degradation products *(13)*. So far, however, no systematic studies have been focused on the degradation of carbohydrates during steam explosion. Because the yield of intact carbohydrate components, sugars and/or polysaccharides, is important for the overall process economics and simplicity, the reaction conditions favoring carbohydrate degradation into HMF and furfural must be known.

In earlier studies, the carbohydrate composition after steam explosion of aspen wood was thoroughly analyzed *(1,5,12)*. Quantitative analysis of the steam-exploded material is, however, difficult because the process is highly heterogeneous *(1)* and several of the products, notably HMF, furfural, and acetic acid, are volatile. A complete mass balance has been difficult to obtain. Because the autohydrolysis products of steam explosion and those obtained from steam treatment are similar *(14)*, the chemistry encountered in these processes can be regarded as interchangeable *(15)*. Steam treatment on wood meal can thus be conveniently carried out in a sealed autoclave, which gives a more homogeneous reaction and permits the recovery of all products regardless of their volatility.

In the present work, aspen wood meal was subjected to heat treatment in order to elucidate the mechanism of steam explosion, the relationship between the degree of carbohydrate depolymerization and the steam acidity, and the effects of an alkaline pretreatment of the wood. Enzymatic hydrolysis by cellulolytic enzymes followed by glucose determination was used to evaluate the accessibility of cellulose in the solid residue after each steam treatment method. The influence of particle size after steam explosion as well as of a lignin removal by extraction prior to enzymatic hydrolysis was also evaluated.

Table 1
Steam Explosion Conditions and Calculated Severity During Treatment of Aspen Wood, Together With Results of Size Screening Into Pulp and Shives Fractions

				After screening	
Sample no.	Temperature (°C)	Time (min)	Severity factor[a]	Shives fraction (%)	Pulp fraction (%)
1	185	5	3.2	—	—
2	205	5	3.8	76.6	23.4
3	200	15	4.1	63.5	36.5

[a]See Eq. 1.

Materials and Methods

Aspen Wood and Equipment

For steam explosion experiments, chips of aspen wood (*Populus tremula*) were homogenized in a Wiley mill using a 10-mm sieve. For the steam treatment experiments, the chips were ground using a 40-mesh sieve. Celluclast, a culture filtrate from a filamentous fungus containing a complete cellulase system, was obtained from Novo Nordisk (Denmark). An almond β-glucosidase (EC 3.2.1.21) was obtained from Sigma (St. Louis, MO). For high-performance liquid chromatography (HPLC) analysis, a Waters system with two Waters 510 pumps, a Waters 717 plus Auto-sampler, a Waters Model 996 photodiode array detector, and Millennium 32 software for operation control and data processing were used.

Steam Explosion Experiments

Steam explosion experiments were performed as described in ref. *1*. Two hundred grams (oven dry, o.d.) of milled wood chips was placed in an autoclave with a volume of 5 L. High-temperature steam was applied to reach 185–205°C. When the time (5–15 min) according to the required severity factor (S_0) was reached (Table 1), a valve was opened and the treated chips were "exploded." The samples were further used for experiments with enzymatic hydrolysis.

Screening of Steam-Exploded Wood

Steam-exploded wood fibers were fractionated using an NAF defibrator with a screen size of 2 mm. The fibers were suspended in water and driven by pressurized water (3 bars) to pass through the screen and collected as the pulp fraction. The rejected fiber material was collected as the shives fraction.

Extraction of Lignin From Steam-Exploded Wood

Soxhlet extraction for 6 h with dioxane:water (9:1) was used to extract lignin from the pulp fraction after screening of the exploded wood. The residue was air-dried.

Table 2
Temperature, Duration, and Calculated Severity for Steam Treatment of Aspen Wood Meal

Sample no.	Temperature (°C)	Time (min)	Severity factor[a]
1	152	15	2.7
2	173	15	3.3
3	156	360	4.2
4	173	360	4.7

[a]See Eq. 1.

Steam Treatment Experiments

Wood meal (4 g o.d.) was placed in a 100-mL autoclave together with 5 mL of deionized water. After sealing, the autoclave was thoroughly flushed and filled with nitrogen to remove all oxygen. The rotating autoclave was heated at either 152 or 173°C for various times in the interval of 15–360 min until the desired S_0 was reached (Table 2). After cooling, the sample was collected on a glass filter and washed with water. The combined water extract was analyzed by acid–base titration to determine the concentration of released acids and by HPLC to determine the contents of HMF and furfural. After drying and weighing, the residue was hydrolyzed (according to the standard method Tappi T222-om-83) and the content of Klason lignin determined. The carbohydrates in the hydrolysate were directly quantified using a newly developed and highly reproducible reverse-phase (RP)-HPLC method after conversion of the sugars to 2,4-dinitrophenylhydrazones (method to be published elsewhere). The steam-treated materials were further used for experiments with enzymatic hydrolysis.

Quantification of Furfural and HMF

HPLC separation was carried out on an ODS column (HICHROM H5ODS-3519) with a size of 4.6 × 150 mm. An isocratic mobile phase of water:acetonitrile (70:30 [v/v]) was used with a flow rate of 1 mL/min. Detection was by ultraviolet (UV) absorption.

Pretreatment With NaOH

Wood meal (4 g o.d.) was suspended in 100 mL of ethanol containing 200 mg of NaOH. After stirring, the wood meal was air-blown to dryness at room temperature before being subjected to steam treatment and analyses as described under Steam Treatment Experiments.

Enzymatic Hydrolysis

A 2-g (o.d.) sample of steam-treated or steam-exploded wood material was suspended in 40 mL of 0.04 *M* acetate buffer at pH 5.0. Celluclast (1000 NCU) was added together with β-glucosidase in a 1:1 ratio. Hydroly-

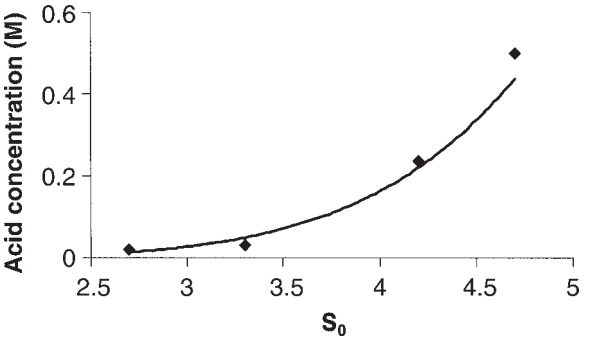

Fig. 1. Formation of acids from steam treatment of aspen wood meal as function of S_0.

sis was carried out at 40°C with continuous stirring of the suspension by magnetic stirring (Fig. 6) or rotation (Fig. 8) to effect efficient mixing. Aliquots of 0.5 mL were taken regularly for carbohydrate analysis using the HPLC method mentioned in the Steam Treatment Experiments section. To evaluate the efficiency of the enzymatic hydrolysis, a fully bleached birch kraft pulp was subjected to the same hydrolysis conditions. Complete hydrolysis was achieved within 12 h.

Results and Discussion

Yield Loss on Steam Treatment

To compare the different degrees of steam treatment and steam explosion used, the S_0 was employed. This factor takes into account the time and temperature and is calculated as follows *(16)*:

$$S_0 = \log\{\exp[(T - 100)/14.75]t\} \quad (1)$$

in which T is the temperature (°C) and t is the duration of treatment (min).

After steam treatment of aspen wood meal with severity factors from 2.7 to 4.7 (Table 2), the resulting aqueous solution and washing liquor were combined and analyzed to determine the content of acidic products. The data obtained were used to calculate the concentration of acid with respect to the original amount of water used in the steam treatment. The concentration is plotted against the severity factor in Fig. 1. It can be seen that the concentration of acids released increased as the severity factor increased. Based on the structure of the native wood constituents, acetic acid previously bonded to the hemicellulose polymer xylan is assumed to be the predominant product *(17)*. At an S_0 of 4.7, a concentration of 0.5 M acid was found, which corresponds to a level of about 3% of acetyl groups in the original wood. This value is close to the total content of acetyl groups known to be present in aspen wood *(18)*.

After steam treatment of the wood meal, the residue was hydrolyzed with sulfuric acid following the procedure for determination of Klason lignin. After removal of the lignin, the remaining solution of monomeric

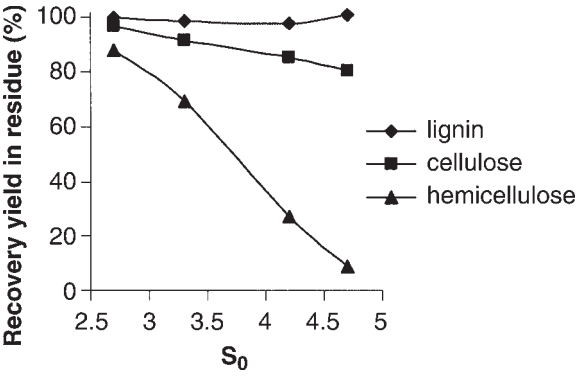

Fig. 2. Yield of lignin, cellulose, and hemicellulose from steam treatment of aspen wood meal as function of S_0.

sugars was reacted with 2,4-dinitrophenyl-hydrazine in order to convert all the sugars into hydrazone derivatives. Subsequently, these were quantified using RP-HPLC. Based on the analytical data, the compositions of lignin, cellulose, and hemicelluloses in the residue from each experiment were calculated (Fig. 2). Although the amount of lignin remained fairly constant, a gradual degradation of the polysaccharides, notably the hemicelluloses, took place with increasing S_0. Both homolytic cleavage and acid hydrolysis of glucosidic linkages may take place during steam treatment *(19)*. Furthermore, linkages between lignin and polysaccharides can also be cleaved *(7)*. As a result, oligomeric and monomeric carbohydrates are formed and solubilized in the aqueous solution, and this reduces the yield of fibrous material. The much greater stability of the cellulose than of the hemicelluloses can be attributed to the high degree of crystallinity and the presence of microfibrils, which decreases accessibility. At the highest S_0 used, S_0 = 4.7, the total yield losses of hemicellulose and cellulose in the solid residue were 91 and 20%, respectively. At the same time, a lignin value slightly above 100% was obtained (Fig. 2). Although this was not investigated further, this indicates that material of carbohydrate origin may have been converted into products with a behavior similar to that of lignin.

Importance of Acid-Catalyzed Degradation of Carbohydrates

Depolymerization of polysaccharides resulting from steam treatment through cleavage of glucosidic linkages is desirable for conversion of carbohydrates through fermentation processes. If, on the other hand, monomeric carbohydrates are further degraded into HMF (from hexoses) and furfural (from pentoses), a true yield loss occurs (Fig. 3). To quantify the extent of such reactions, the aqueous extracts after steam treatment were analyzed by HPLC using a diode array detector, and the chromatograms were compared with those of authentic compounds. Typical three-dimensional (3D) spectra of furfural and HMF are shown in Fig. 4.

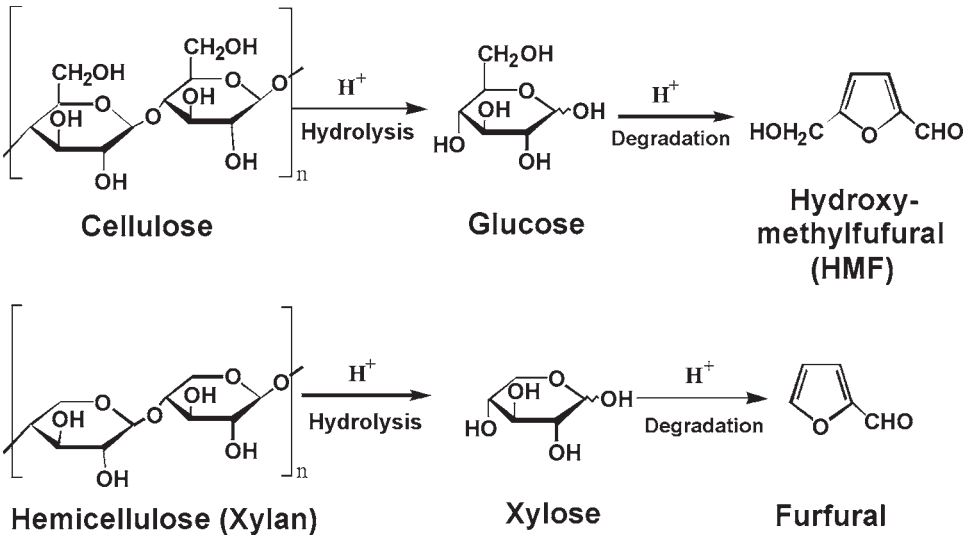

Fig. 3. Reaction sequence for consecutive decomposition and degradation reactions of cellulose and hemicellulose (xylan) during steam treatment.

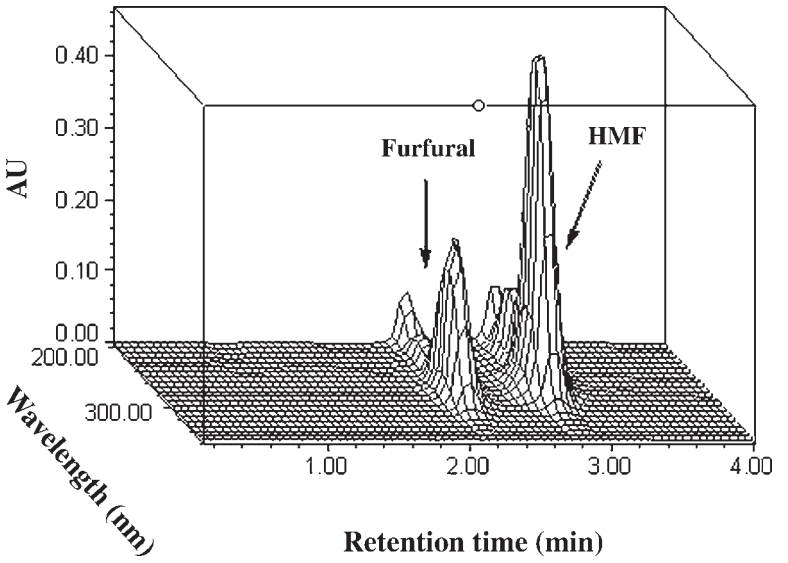

Fig. 4. Typical 3D spectra for HPLC-UV analysis of furfural and HMF. Column: HICHROM H5ODS-3519 with 4.6 × 150 mm i.d.; flow-rate: 1 mL/min; mobile phase: water:acetonitrile (7:3).

This method permitted a direct analysis of the aqueous extracts without any derivatization. In addition, the presence of lignin fragments or other UV-absorbing material could be distinguished from furfural and HMF.

Both furfural and HMF were readily quantified, as shown in Fig. 5. Here, the amounts of furfural and HMF found were calculated as percent-

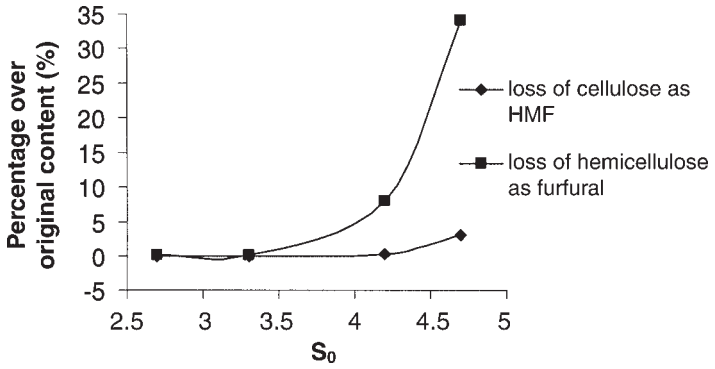

Fig. 5. Formation of furfural and HMF as function of S_0 in steam treatment of aspen wood meal.

ages of the original amounts of hemicellulose (xylan) and cellulose, respectively. The calculations were based on the fact that carbohydrates were the only source of furfural and HMF and that these compounds are formed in a 1:1 ratio from monomeric sugars, as shown in Fig. 3. From a chemical point of view, however, furfural and HMF are neither the only nor the final products of acid conversion of sugars. Furthermore, it has been suggested *(15)* that reactions between these compounds and lignin occur under steam treatment or steam explosion conditions. To assess the stability of furfural and HMF under acidic conditions, a standard mixture of these compounds was treated with 3% sulfuric acid at 120°C for 1 h, i.e., the conditions used in the standard method for Klason lignin determination. Recovery yields of 100 and 80%, respectively, were obtained, demonstrating that the values shown in Fig. 5 for furfural should be regarded as fairly representative. For HMF, on the other hand, a further degradation related to the severity of the steam treatment cannot be excluded.

In Fig. 5, it is obvious that a substantial degradation of carbohydrates occurs on steam treatment in spite of the low acidity. When the S_0 was equal to 4.7, about 35% of the original hemicellulose was converted into furfural while 3% of the cellulose degraded into HMF. When these amounts were recalculated based on the total losses of hemicellulose (91%) and cellulose (20%), as shown in Fig. 2, it was concluded that a minimum of 37% of the hemicellulose and 15% of the cellulose were degraded into furfural and HMF, respectively.

Suppression of Carbohydrate Degradation

The acid-catalyzed degradation of carbohydrates during steam treatment not only leads to a loss of valuable products, but the furfural and HMF formed are known to be toxic and to inhibit the fermentation of sugars *(20)*. Because the formation of furfural and HMF requires acidic conditions, the effect of lowering the steam's acidity by the addition of alkali prior to steam treatment was studied. An amount of NaOH calculated to neutralize all the

acid formed at an S_0 of about 4.7 was added to aspen wood meal followed by steam treatment. The results are shown in Table 3 together with the results of a control experiment, both carried out at an S_0 of 4.6. The presence of alkali produced a much higher final pH (4.5) in the aqueous extract after reaction than in the control (pH 3.1). In addition, the formation of furfural and HMF was almost completely suppressed. As a consequence, the yield of lignin-free residue increased by about 3 percentage units, the predominant part being cellulose. From a mechanistic point of view, the shift toward a higher pH during the steam treatment should lead to a reduced degree of acid hydrolysis in both the polysaccharides and the lignin. Thus, the relative importance of homolytic cleavage reactions may increase *(19)*. As a result, the depolymerization reactions in the chemical conversion of carbohydrates will predominate over the acid-catalyzed degradation reactions (Fig. 3).

Because the yield of residue after the steam treatment was somewhat lower in the experiment carried out in the presence of alkali, a substantial portion of the hemicellulose can be found as partially hydrolyzed oligosaccharide fragments in the aqueous extract *(21)*. Further support for a "cleaner" reaction in the presence of alkali was obtained by extraction of the steam treatment residues with dioxane:water (9:1). Although the control gave a higher amount of extractable material, the remaining content of Klason lignin in this sample was found to be higher, implying a greater amount of "lignin-like" impurities of carbohydrate origin (Table 3).

Factors Affecting Enzymatic Hydrolysis

We investigated several factors that impact the enzymatic hydrolyzability of the pretreated aspen materials. The steam treatment or steam explosion of biomass prepares a cellulosic fraction that can be further processed to fuels or chemical products by chemical or biochemical routes *(22)*. When enzymatic hydrolysis is used for the production of glucose with subsequent fermentation to ethanol by yeasts, the pretreatment should give rise to fibrous material with a large surface area in order to facilitate the enzymatic attack *(23)*. In addition, the treatment conditions should be chosen so that no acid degradation products with a toxic effect on the yeast or the system are created *(24)*. In reality, the steam explosion process results in a highly heterogeneous fibrous product with completely defibrated material present together with fiber bundles and rather unaffected wood shives *(1)*. Furthermore, a rather severe degradation of hemicellulose *(1)* and a change in the structure of lignin takes place *(8)*. To evaluate the importance of these factors for the enzymatic hydrolysis of cellulose, aspen wood samples obtained after steam explosion and after steam treatment were further treated prior to enzymatic hydrolysis. By assessing the formation of glucose, the importance of morphology (i.e., the explosion step), the fiber homogeneity, and the presence of lignin degradation products were evaluated.

Table 3
Effects of NaOH Pretreatment on Steam Treatment of Aspen Wood Meal at S_0 of 4.6 (177°C, 210 min)

	pH	Glucose loss as HMF (%)[a]	Xylose loss as furfural (%)[a]	Solid residue yield (%)[b]	Extraction yield (%)[c]	Lignin content (%)[d]	Lignin-free solid residue (%)[b]
Control	3.11	2.7	31	70.8	21.8	17.8	45.5
Pretreatment with NaOH	4.49	0.003	0.8	68.5	18.1	14.1	48.2

[a]Based on theoretical amount of sugar.
[b]Based on wood meal.
[c]Extraction of residue with dioxane:water (9:1).
[d]As Klason lignin in extracted residue.

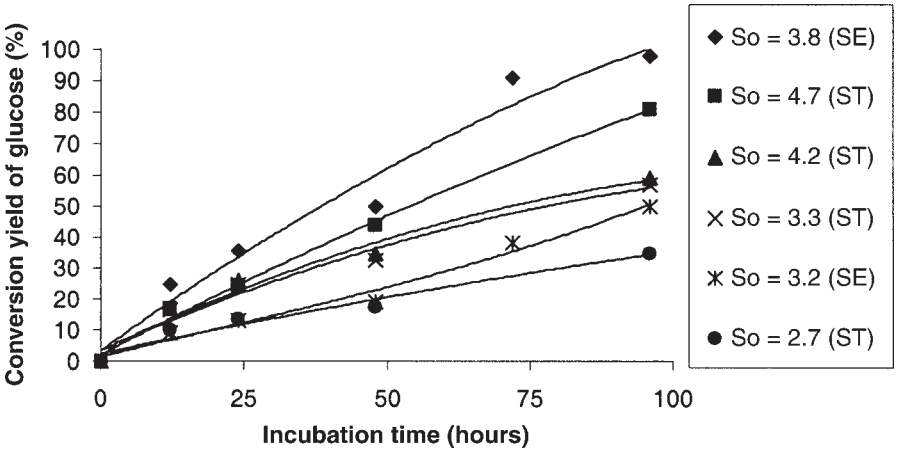

Fig. 6. Yield of glucose as function of enzymatic hydrolysis time for steam-treated (ST) or steam-exploded (SE) aspen wood. The yield of glucose was based on the theoretical amount of glucose in the starting material.

Steam explosion experiments were carried out at severity factors in the range of 3.2–4.1 (Table 1), and steam treatment experiments with severity factors of 2.7–4.7 (Table 2). On enzymatic hydrolysis using the Celluclast cocktail supplemented with β-glucosidase, glucose was continuously formed, as shown in Fig. 6. The amount of enzyme-released glucose was related to the total amount of glucose present in each sample (determined by a standard method after complete acid hydrolysis). The data in Fig. 6 indicate that although the steam explosion was performed on aspen wood particles (<10 mm) whereas the steam treatment employed wood meal (<40 mesh), the former gave an equal or more efficient formation of glucose. Obviously, the sudden release of pressure in the "explosion" results in a physical rupture of the fiber walls that is beneficial to the accessibility of the enzymes. These data indicate that rather than increasing the acidity in the process, increasing the pressure change might be more beneficial.

Because fibrous materials after steam explosion were very heterogeneous, a laboratory fractionation using an NAF defibrator was employed to obtain more homogeneous fractions, one pulp fraction and one shives fraction (Fig. 7). Such a fractionation was done after two of the steam explosion experiments (Table 1). On both of these, enzymatic hydrolysis with the Celluclast cocktail was carried out and, again, the formation of glucose was monitored. In additional experiments, the influence of removing dioxane-soluble materials (such as lignin) on the enzymatic hydrolysis of the resulting solids was investigated (Fig. 7). The results shown in Fig. 8 demonstrate that there are large differences in digestibility between the pulp and the shives fractions when compared at a common S_0 value. Furthermore, a preextraction with dioxane:water (9:1), resulting in a reduction in the Klason lignin content by about 50%, was found to increase the amount of glucose compared with that in pulp fractions obtained from the same steam

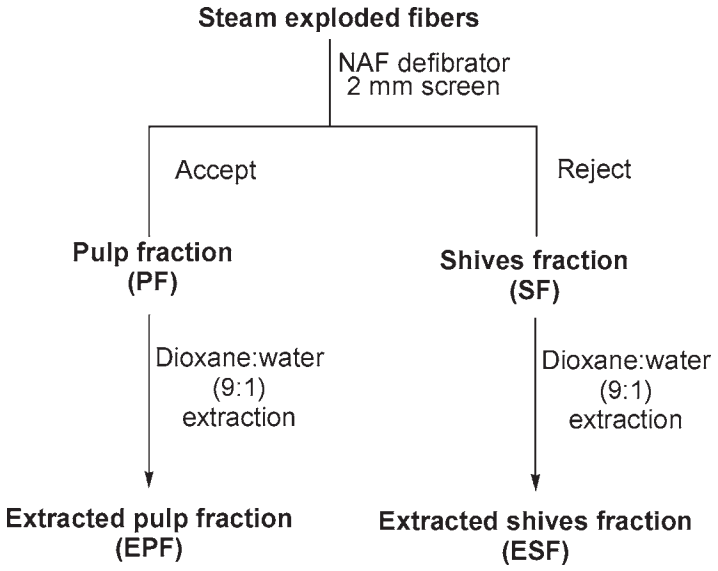

Fig. 7. Experimental diagram for NAF fractionation and dioxane:water extraction of steam-exploded fibers before enzymatic hydrolysis.

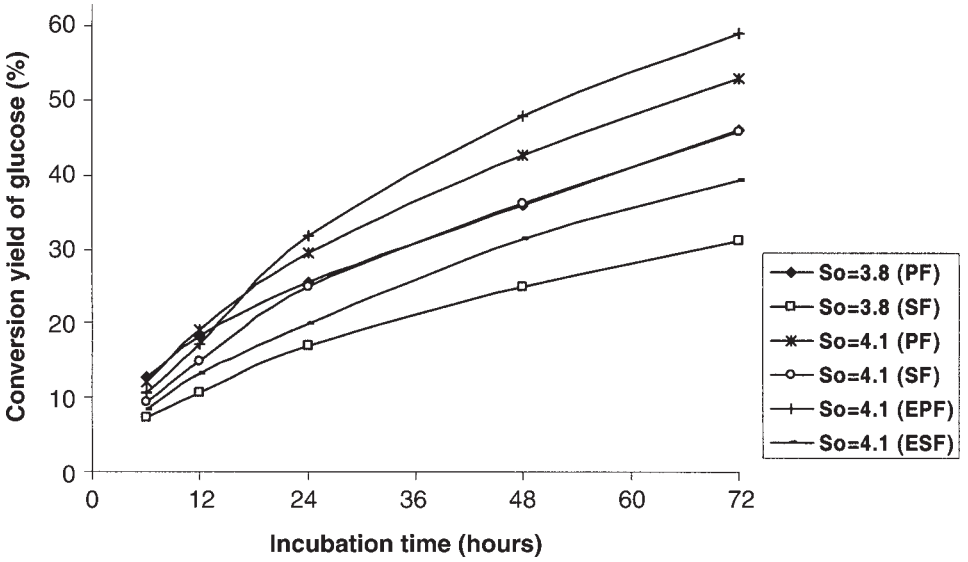

Fig. 8. Yield of glucose as function of enzymatic hydrolysis time for steam-exploded aspen wood. PF, pulp fraction; SF, shives fraction; EPF, dioxane:water (9:1)–extracted PF; ESF, dioxane:water (9:1)–extracted SF. The yield of glucose was based on the theoretical amount of glucose present in the steam-exploded solid material.

explosion experiment. The results obtained from the shives fractions with and without preextraction were, however, contradictory and gave the opposite result. From the curves shown in Fig. 8, it is, however, obvious that

the enzymatic hydrolysis may be affected by other interactions. The rate of formation of glucose was found to differ among samples, in particular at short incubation times (<24 h). In addition to the homogeneity of the material, the presence or absence of nonextractable degradation products from lignin or carbohydrates able to interact with the enzyme can be assumed to play a role *(24)*.

Conclusion

During steam treatment or steam explosion, homolytic cleavage as well as acid hydrolysis of glucosidic linkages in polysaccharides takes place. The sugars released are further converted into furfural and HMF, causing a severe yield loss that is dependent on the severity of the treatment. Pretreatment of the wood with alkali results in a much reduced formation of sugar degradation products, and it is mainly the depolymerization of the polymers that occurs through homolytic cleavage of glucosidic linkages. Steam explosion gives carbohydrates with a better enzymatic digestibility than steam treatment, owing to the greater accessibility of the cellulose toward enzymes. Fractionation of the steam-exploded material and the elimination of extractable degradation products further improve the accessibility.

Acknowledgments

We thank Tomas Josefsson for assistance with the steam explosion experiments. We gratefully acknowledge the Swedish National Energy Administration for financial support.

References

1. Josefsson, T., Lennholm, H., and Gellerstedt, G. (2002), *Holzforschung* **53(3),** 289–297.
2. Law, K. N., Zhou, W. X., and Valade, J. L. (1990), *J. Pulp Paper Sci.* **16(6),** J184–J189.
3. Law, K. N., Zhou, W. X., and Valade, J. L. (1991), *TAPPI J.* **4,** 263–267.
4. Zhan, H. and Chen, J. (1994), *Cellulose Chem. Technol.* **28,** 329–338.
5. Josefsson, T. (2001), PhD thesis, Department of Pulp and Paper Chemistry and Technology, Royal Institute of Technology, Stockholm, Sweden.
6. Schwald, W., Brownell, H. H., and Saddler, J. N. (1988), *J. Wood Chem. Technol.* **8,** 543–560.
7. Avellar, B. K. and Glasser, W. G. (1998), *Biomass Bioenergy* **14,** 205–218.
8. Robert, D., Bardet, M., Lapierre, C., and Gellerstedt, G. (1988), *Cell. Chem. Technol.* **22,** 221–230.
9. Lora, J. H. and Wayman, M. (1979), *TAPPI J.* **61(6),** 47–50.
10. Robert, D., Gellerstedt, G., and Bardet, M. (1986), *Nordic Pulp Pap. Res. J.* **1,** 18–25.
11. Wayman, M. and Lora, J. H. (1978), *TAPPI J.* **61,** 55–57.
12. Josefsson, T., Lennholm, H., and Gellerstedt, G. (2001), *Cellulose* **8,** 289–296.
13. Pigman, W. W. and Geopp, R. M. J. (1948), *Chemistry of Carbohydrates*, Academic, New York.
14. Kosikova, B., Ebringerova, A., and Kacurakova, M. (1995), *Cell. Chem. Technol.* **29,** 683–690.
15. Jakobsons, J., Hortling, B., Erins, P., and Sundquist, J. (1995), *Holzforschung* **49,** 51–59.
16. Overend, R. P. and Chornet, E. (1987), *Philos. Trans. R. Soc. Lond.* **A321,** 523–536.

17. Subramanian, R. V., Somasekharan, K. N., and Johns, W. E. (1983), *Holzforschung* **37,** 117–120.
18. Sjöström, E., Janson, J., Haglund, P., and Enström, B. (1965), *J. Polym. Sci.* **11,** 221–241.
19. Iwanade, A., Ohi, H., and Yamaguchi, A. (1995), *Mokuzai Gakkaishi* **41,** 683–688.
20. Larsson, S., Palmqvist, E., Hahn-Hägerdal, B., Tengborg, C., and Stenberg, K. (1999), *Enzyme Microb. Technol.* **24,** 151–159.
21. Zhuang, Q. and Vidal, P. F. (1997), *Cell. Chem. Technol.* **31,** 37–49.
22. Lipinsky, E. S. (1979), *Adv. Chem. ACS series* 181, 1–21.
23. Himmel, M. E., Baker, J. O., and Saddler, J. N. (2000), ACS Symposium Series No. 769, Washington, DC, American Chemical Society.
24. Clark, T. A. and Mackie, K. L. (1984), *Chem. Technol. Biotechnol.* **34B,** 101–110.