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Combined Steam Pretreatment and Enzymatic Hydrolysis of Starch-Free Wheat Fibers

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Abstract

Steam treatment of an industrial process stream, denoted starch-free wheat fiber, was investigated to improve the formation of monomeric sugars in subsequent enzymatic hydrolysis for further bioconversion into ethanol. The solid fraction in the process stream, derived from a combined starch and ethanol factory, was rich in arabinose (21.1%), xylose (30.1%), and glucose (18.6%), in the form of polysaccharides. Various conditions of steam pretreatment (170–220°C for 5–30 min) were evaluated, and their effect was assessed by enzymatic hydrolysis with 2 g of Celluclast + Ultraflo mixture/100 g of starch-free fiber (SFF) slurry at 5% dry matter (DM). The highest overall sugar yield for the combined steam pretreatment and enzymatic hydrolysis, 52 g/100 g of DM of SFF, corresponding to 74% of the theoretical, was achieved with pretreatment at 190°C for 10 min followed by enzymatic hydrolysis.

Index Entries: Wheat fibers; steam pretreatment; enzymatic hydrolysis; starch-free fiber; microwave oven.

Introduction

In the production of starch from wheat, several byproduct streams are obtained *(1)*. The starch-free fiber (SFF) stream used in the present investigation originated from a combined starch and ethanol factory. Currently, this SFF stream is marketed as low-cost animal feed. Hemicellulose and glucan (cellulose) are the major components of this stream, comprising about 70% of its dry weight. Recovery of the monomeric sugars that compose this material (i.e., xylose, arabinose, and glucose) by performing some sort of hydrolysis would increase the amount of sugars available for ethanol production. Thus, utilization of this stream in the

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very same plant where it is generated and where ethanol is already produced could decrease the ethanol production cost, since the cost of the feedstock is one of the major contributors to the relatively high production cost of fuel ethanol *(2)*.

In a previous investigation on another batch of SFF, the optimal conditions for pretreatment were determined using heat treatment in a microwave oven followed by enzymatic hydrolysis. The severity factor, $\log(R_o)$, which combines the temperature and residence time parameters, was used to compare the results at different conditions:

$$\mathrm{Log}\left(R_o\right) = \mathrm{Log}\left\{t \cdot \exp\left[\frac{\left(T_r - T_b\right)}{14.75}\right]\right\} \quad (1)$$

in which t is the reaction time (min); T_r is the reaction temperature (°C); and T_b is the reference temperature, which is set to 100°C (3). A severity factor of 3.65 resulted in the highest total sugar yield (4). In the present study, the same pretreatment was repeated on the new SFF batch to determine whether there were any differences in the materials. Combined acid hydrolysis in a microwave oven and enzymatic hydrolysis was performed mainly to allow analysis of the maximum amount of carbohydrates available. Because pretreatment using microwave irradiation is not feasible on a large scale, steam pretreatment was investigated as a means of pretreatment of SFF on an industrial scale. This method, consisting of high-pressure steaming, remains one of the best ways of increasing the enzyme digestibility of fibers and is regarded as one of the key technologies in the cost-effective manufacture of bioethanol *(2,5–7)*. The effect of enzyme loading on the direct enzymatic hydrolysis of SFF and on enzymatic hydrolysis following pretreatment was also investigated.

Materials and Methods

Figure 1 shows the experimental procedure used to evaluate the hydrolysis methods of SFF. First, the SFF material was subjected to saccharification with amyloglucosidase to determine the amount of starch available in the material. Second, the material was subjected to pretreatment in either a steam pretreatment unit or a microwave oven, followed by enzymatic hydrolysis. Pretreatment in a microwave oven was performed both with and without the addition of acid. Direct enzymatic hydrolysis was also performed.

Raw Material

Wheat SFF material was kindly donated by Amylum UK (Nesle, France). The material had a dry matter (DM) content of 18.5% and was stored in buckets at –18°C. The composition, analyzed by combined acid hydrolysis with 1% H_2SO_4 followed by enzymatic hydrolysis with cellulolytic and hemicellulolytic enzymes, is given in Table 1.

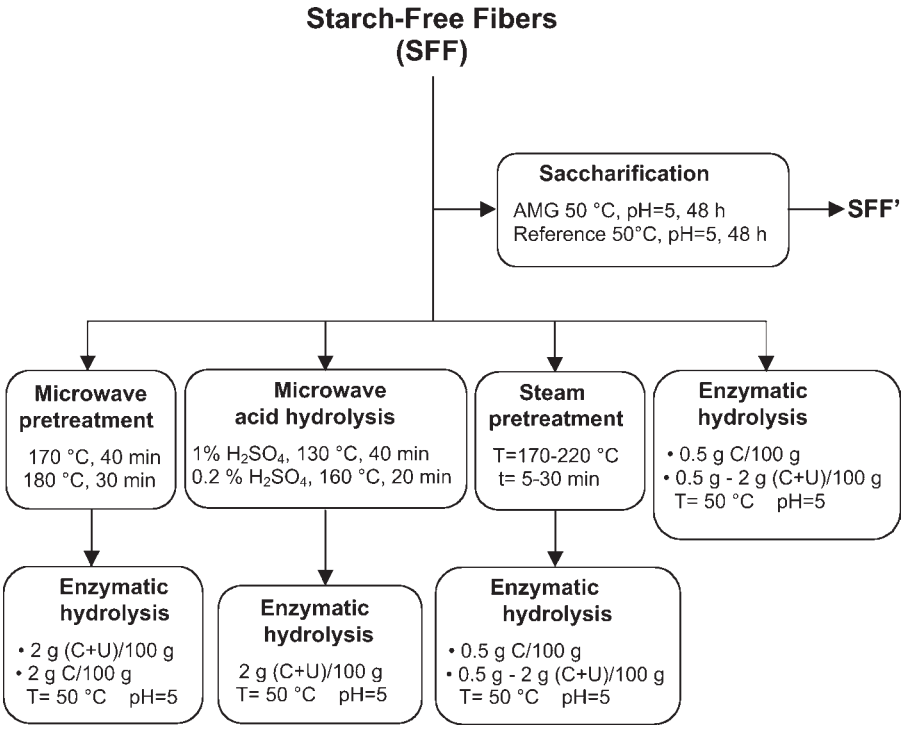

Fig. 1. Experimental procedure used for assessment of various methods for the hydrolysis of SFFs.

Table 1
Composition of SFF Material

	g/100 g SFF (dry wt basis)
Arabinose [a]	21.1
Xylose [a]	30.1
Glucose [a]	18.6
Galactose [a]	2.0
Acid-soluble lignin [b]	4.0
Acid-insoluble lignin [c]	15.2
Ash [d]	1.1
Total	92.1

[a] Determined by acid hydrolysis with 1% H_2S0_4 at 130°C for 40 min followed by enzymatic hydrolysis with 2 g of Celluclast + Ultraflo/100 g of slurry (5% DM).

[b] NREL standard method for determination of acid-soluble lignin (LAP-004).

[c] NREL standard method for determination of acid-insoluble lignin (LAP-003).

[d] NREL standard method for determination of ash (LAP-005).

Saccharification

The SFF was saccharified with amyloglucosidase (AMG 300L; Novozymes A/S, Bagsvaerd, Denmark) to determine the starch (or dextrin) content. The material was diluted to an 8 wt% DM slurry, the pH was adjusted to 5.0 and AMG was added at a concentration of 1.2 mL/100 g of SFF slurry. Enzymatic hydrolysis was carried out using a 1-L Rotavapor (Büchi Labortechnik AG, Flawil, Switzerland) containing 800 g of slurry, at 50°C for 48 h. A reference SFF slurry not containing AMG was subjected to the same conditions. The liquid fractions were analyzed regarding glucose, arabinose, xylose, and galactose contents.

Steam Pretreatment

Pretreatment was performed in a steam pretreatment unit. This equipment consists of a 2.4-L vessel into which steam from a boiler saturated up to 40 bar is introduced. The saturated steam permeates the SFF material and causes a high internal pressure. After a certain time, the pressure is suddenly released by opening a valve in the reactor to atmospheric pressure. The rapid change in pressure leads to expansion of the cellulose/hemicellulose structure. The heat treatment provides partial hydrolysis, which, in combination with the expansion following decompression, improves the action of enzymes. Pretreated material is collected in a cyclone connected to the outlet of the pressurized vessel. A computer, ABC 800 (Luxor AB, Motala, Sweden), is used to control valve operation and to record the temperature. The pretreatment vessel was preheated with steam prior to loading 1 kg of the original SFF material (18.5% DM), corresponding to approx 2 L in volume. The material was heated by steam to the desired temperature, and when the preset pretreatment time had elapsed, the material was discharged into the cyclone. Then steam was introduced into the reactor for a short period of time (usually 30 s) at the same temperature to rinse out any remaining material. The time required to attain the desired temperature was approx 20 s. Temperatures of 170, 180, 190, 200, 210, and 220°C and times of 5, 10, 15, 20, and 30 min, were investigated (Table 2).

The liquid fraction of the pretreated material was analyzed for glucose, arabinose, xylose, galactose, furfural, and hydroxymethylfurfural. The slurry was further hydrolyzed with enzymes.

Heat Treatment in Microwave Oven

A microwave oven, MLS-1200 Mega Microwave workstation, from Milestone (Sorisole, Italy), described in a previous study *(4)*, was used to pretreat a 5% DM SFF slurry. Heat treatment was performed at 170°C for 40 min and at 180°C for 30 min. Acid hydrolysis was performed with 1% H_2SO_4 at 130°C for 40 min and with 0.2% H_2SO_4 at 160°C for 20 min. These pretreatment experiments were performed to allow analysis of the material, as well as to compare the SFF material with that used in a previous investigation *(4)*.

Table 2
Experimental Conditions in Pretreatment Step Performed with Steam Gun

Run no.	Severity (Log[R_o])	Temperature (°C)	Time (min)	DM content after pretreatment (%)[a]
1	3.24	170	15	10.2
2	3.35	190	5	12.4
3	3.36	170	20	9.8
4	3.36	180	10	10.5
5	3.53	180	15	9.3
6	3.64	200	5	12.2
7	3.65	190	10	10.5
8	3.66	180	20	9.3
9	3.83	180	30	9.1
10	3.94	200	10	10.1
11	3.94	210	5	10.5
12	3.95	190	20	8.4
13	4.23	220	5	9.8
14	4.24	210	10	11.3
15	4.53	220	10	9.8

[a] DM includes material recovered by rinsing steam.

Enzymatic Hydrolysis

The hemicellulose-degrading enzymes (Ultraflo L) and the cellulases (Celluclast 1.5L) were kindly donated by Novozymes A/S. Celluclast 1.5L had a filter paper activity of 80 filter paper units/mL, determined according to the procedure of Mandels *(8–9)*. For the present study the most important side activities of Ultraflo L were endo-1,4-β-xylanase, β-xylosidase, and α-arabinofuranosidase *(10)*.

The DM content of the steam-pretreated SFF was adjusted to 5% and the pH set to 5.0 with NaOH. Enzymatic hydrolysis was performed using a Celluclast + Ultraflo mixture (1:1) at a ratio of 2 g of enzyme/100 g of slurry *(10)*. Hydrolysis was carried out for 72 h in 100-mL shake flasks maintained at 50°C and shaken at 200 rpm in a laboratory rotary shaker-incubator (LSR/L-V; Adolf Kühner AG) for 72 h. Samples were withdrawn after 0, 2, 4.5, 7.5, 11, 14, 24, 31, 38, 48, 60, and 72 h for analysis of monosaccharides. Direct enzymatic hydrolysis of a 5% DM SFF slurry was also performed as a reference to evaluate the effect of steam pretreatment on the yield.

Enzyme Loadings

Pretreatment was applied at three levels of severity—220°C for 5 min, Log(R_o) = 4.23; 190°C for 10 min, Log(R_o) = 3.65; and 170°C for 15 min, Log(R_o) = 3.24—for enzymatic hydrolysis with the addition of 0.5 g of Celluclast + Ultraflo mixture/100 g of SFF slurry and 0.5 g of Celluclast/100 g of SFF slurry.

Analysis

The DM content of the samples was determined by drying duplicate samples for 16 h in an oven at 105°C according to the National Renewable Energy Laboratory (NREL) standard method for determination of total solids in biomass, LAP-001 *(11)*. Total ash content was determined by ashing samples in triplicate at 575°C in a muffle furnace according to the NREL standard method for ash in biomass, LAP-005 *(12)*.

Samples for high-performance liquid chromatography analysis were withdrawn from the liquid fraction of the SFF slurry after starch hydrolysis, heat pretreatment, acid hydrolysis, and enzymatic hydrolysis. All samples were analyzed regarding arabinose, galactose, glucose, and xylose content using a high-performance liquid chromatography (Shimadzu, Kyoto, Japan) equipped with a refractive index detector (Shimadzu). Separation was performed using an Aminex HPX-87P column (Bio-Rad, Hercules, CA) at 80°C with deionized water at a flow rate of 0.5 mL/min as the mobile phase. The contents of furfural and hydroxymethylfurfural (HMF) were determined in the samples taken after heat pretreatment and after acid hydrolysis of the SFF slurry. Furfural and HMF were separated on an Aminex HPX-87H column (Bio-Rad) at 65°C using 5 m*M* H_2SO_4 as the mobile phase at a flow rate of 0.5 mL/min. All samples were filtered through 0.2-µm filters before analysis.

Soluble and insoluble lignin were determined with the NREL standard biomass analytical methods for the analysis of acid-insoluble and acid-soluble lignin in biomass, LAP-003 and LAP-004, respectively *(13–14)*. NREL's laboratory analytical procedure for the determination of carbohydrates in biomass, LAP-002, without correction for hydrolysis losses was performed to determine the sugar content *(15)*.

Results and Discussion

The material denoted SFF investigated, was the solid fraction obtained after liquefaction of wheat starch fibers with α-amylase and subsequent separation and washing from the glucose syrup. This procedure was carried out at the wheat-milling industry providing the material. Table 1 shows the composition of the SFF material. All values are given on a DM basis. Carbohydrates, determined by acid hydrolysis with 1% H_2SO_4 followed by enzymatic hydrolysis, comprised about 70 wt% of the SFF. These sugar values agreed well with the values determined with the NREL standard analytical method for the determination of carbohydrates, consisting of two-stage acid hydrolysis. The latter resulted in slightly lower sugar values: 28.4 g of xylose, 20.9 g of arabinose, and 18.1 g of glucose/100 g of SFF.

Analysis showed high values of lignin content, which may comprise compounds formed by the degradation of monosaccharides, as well as other compounds. No references in the literature have been found. The raw material also contained a small amount of ash, equal to 1.1 g/100 g of SFF. The analyzed compounds accounted for 92.1% of the DM.

Table 3
Sugar Yields After Pretreatment and Acid Hydrolysis
Performed in Microwave Oven Followed by Enzymatic Hydrolysis

	Yield (g/100 g of SFF)			
	Arabinose	Xylose	Glucose	Total
Pretreatment				
170°C, 40 min + EH	10.8	17.7	17.9	46.4
	7.9	**12.0**	**15.6**	**35.5**
180°C, 40 min + EH	10.7	18.4	18.5	47.6
	6.2	**11.4**	**15.0**	**32.6**
Acid hydrolysis				
0.2% H_2SO_4, 160°C, 20 min + EH	16.5	26.1	18.1	60.7
	6.8	**16.3**	**14.1**	**37.2**
1% H_2SO_4, 130°C, 40 min + EH	21.1	30.1	18.6	69.8
	7.2	**17.4**	**14.1**	**38.7**

[a]EH, enzymatic hydrolysis. The values in boldface are from a previous investigation *(4)* on similar material and are given for comparison.

No starch saccharification was performed on the SFF material used in this study, and therefore some starch-glucose could still be present as dextrins (malto-dextrins) in the material. For this reason, saccharification with AMG was performed in order to determine the starch-glucose still present in the material. The results showed that only 2 g of glucose/100 g DM of SFF was released during saccharification. It was thus decided that starch hydrolysis of the SFF prior to steam pretreatment to hydrolyze the hemicellulose/cellulose was unnecessary. Surprisingly, 3 g of xylose and 2 g of arabinose were also released per 100 g of SFF during hydrolysis with AMG. To investigate whether this depended on the heat treatment (50°C for 48 h) or was owing to the AMG preparation, a further experiment was carried out using the same pH and temperature conditions for 48 h but without the addition of AMG. The results showed that no sugar was released when the SFF material was subjected to hydrolysis conditions without the addition of enzyme. Thus, it appears that the AMG preparation also contains some hemicellulolytic activity.

Comparison of Two Different Batches

Table 3 shows the sugar yields obtained after pretreatment and enzymatic hydrolysis of the SFF material used in the present study and the SFF material utilized in a previous study (boldface in Table 3) *(4)*. The sugar yields obtained with acid hydrolysis in a microwave oven followed by enzymatic hydrolysis are also given. Unless otherwise stated, the yields following pretreatment and enzymatic hydrolysis are expressed as g/100 g of dry SFF. Galactose was present at very low amounts in both materials.

Therefore, only xylose, arabinose, and glucose were used for evaluation of the yields. These two batches were collected at different times and stages of the process at the starch factory, so some difference in composition could therefore be expected.

The material used in the present study contained higher amounts of carbohydrates. The higher sugar content can, to some extent, be ascribed to the fact that the material was withdrawn before the saccharification step in the factory, in contrast to the old material. As already mentioned, starch saccharification releases some pentoses as well. If these monosaccharides, released in the saccharification step, are subtracted from the sugar yields obtained with the new material, the difference in yield between the two materials diminishes. However, the new material still has a higher amount of carbohydrates.

Steam Pretreatment Followed by Enzymatic Hydrolysis

Pretreatment was performed in a steam pretreatment unit in which the SFFs were exposed to steam at high pressure. Owing to continued introduction of steam into the reactor during steam pretreatment, the SFFs are diluted, resulting in a slurry with lower DM content, between 9 and 12% (*see* Table 2), than the initial material, 18.5%.

The total yield of (fermentable) sugars after both pretreatment and combined pretreatment and enzymatic hydrolysis is shown in Fig. 2. Steam pretreatment did not have a significant effect on the sugar yield but enhanced the following enzymatic hydrolysis. The steam pretreatment that resulted in the highest release of monomer sugars, 6.4 g of total sugars/100 g, was 200°C and 10 min, which corresponds to a severity factor of 3.94. Steam pretreatment at 190°C for 10 min (Log[R_o] = 3.65) resulted in the highest total sugar yield, 51.8 g/100 g, after subsequent enzymatic hydrolysis. This yield, which consists of 13.4 g of arabinose, 21.2 g of xylose, and 17.2 g of glucose, corresponds to 74.2% of the theoretical. However, similar yields, between 44 and 49 g/100 g, were achieved over the entire range of pretreatment conditions tested. The main improvement in steam pretreatment at 190°C for 10 min was seen on the glucose yield, which increased to 92.4% of the theoretical, while the yield of pentoses only increased to 69.4 and 55.5% for xylose and arabinose, respectively. A severity factor of 3.65 proved to be optimal in obtaining the maximum total sugar yield by heat pretreatment followed by enzymatic hydrolysis, using either a microwave oven (at 170°C for 40 min) or a steam pretreatment unit (at 190°C for 10 min).

Figure 3 shows the yield of individual sugars after combined pretreatment and enzymatic hydrolysis. Most of the arabinose was released at low severity (severity factors between 3.3 and 3.7). The highest arabinose yield, about 13.5 g/100 g of SFF, was obtained at 170°C, 15 min; 170°C, 20 min; 180°C, 15 min; and at 190°C, 10 min. At lower severity, somewhat less arabinose was released, while higher severities resulted in partial degradation. A severity factor of about 3.65 was required to obtain the maximum yield of xylose, reaching a yield of 21.2 g/100 g of SFF, at 190°C for 10 min.

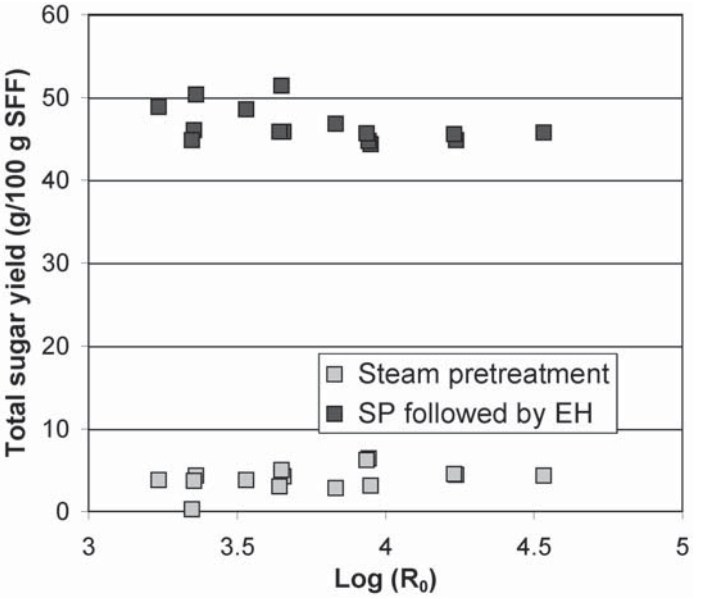

Fig. 2. Effect of severity factor (Log[R_o]) on total sugar yield after steam pretreatment (SP) and after combined SP and enzymatic hydrolysis (EH).

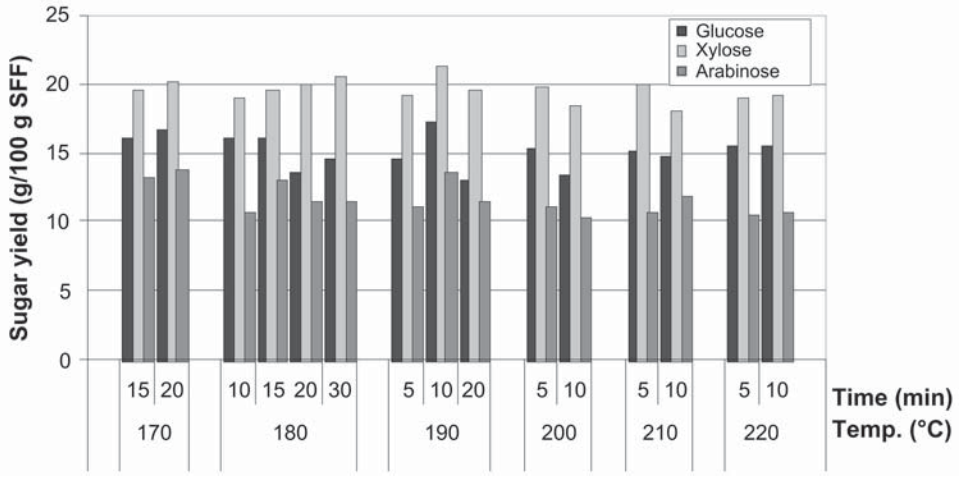

Fig. 3. Effect of pretreatment conditions on yield of individual sugars after combined steam pretreatment and enzymatic hydrolysis.

The same conditions also resulted in the maximum glucose yield, 17.2 g/100 g of SFF. At the pretreatment conditions used, the formation of fermentation-inhibiting compounds was very low (results not shown) *(16)*. The concentration of furfural in the slurry pretreated at 190°C for 10 min was about 0.3 g/L. No HMF was detected.

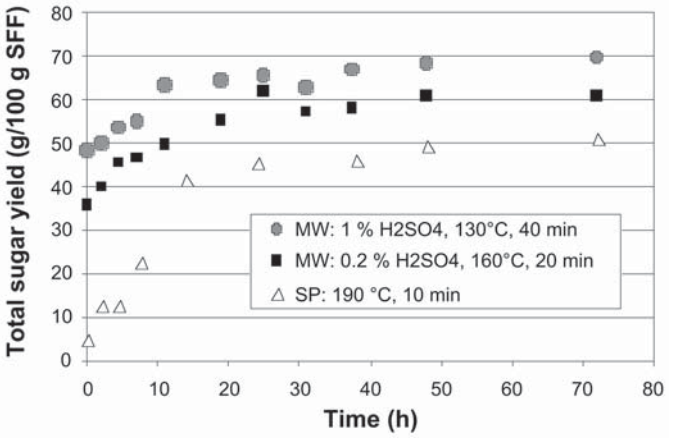

Fig. 4. Sugar yields as a function of enzymatic hydrolysis time. Comparison of best combination of pretreatment and enzymatic hydrolysis methods using either microwave oven (MW) or steam pretreatment (SP).

Further comparison of the maximum yields obtainable with the different hydrolysis methods showed that the addition of acid during pretreatment substantially increased the total sugar yield (Fig. 4). The analytical method consisting of combined acid hydrolysis with 1% H_2SO_4 at 130°C for 40 min followed by enzymatic hydrolysis released the highest amount of total sugars, corresponding to about 70 wt% of the total DM. This value was taken as the maximum achievable, i.e., 100% of theoretical. Direct acid hydrolysis with 1% H_2SO_4 resulted in 69% of the theoretical. The concentrations of furfural and HMF were 0.15 and 0.01 g/L, respectively.

Acid hydrolysis with 0.2% H_2SO_4 at 160°C for 20 min resulted in 50% of the theoretical sugar yield. This is approximately the same yield as that obtained with direct enzymatic hydrolysis. When the acid hydrolysis was followed by enzymatic hydrolysis, the sugar yield increased to 88% of the theoretical. Analysis of furfural and HMF showed that only 0.2 g/L of furfural was present in the slurry and no HMF was formed.

Besides two different hydrolysis methods (i.e., acid hydrolysis and pretreatment without the addition of acid), two different pieces of pretreatment equipment were used to perform the experiments (Fig. 4). Acid hydrolysis was conducted in a microwave oven, while pretreatment was performed in a steam pretreatment unit. The microwave oven provides a closed system where the amount of water added is fixed and there is no loss of material during the process *(17–18)*. On the other hand, the samples have to be rather diluted for the microwave oven to be efficient. Another disadvantage is that the microwaves penetrate the material only a few centermeters, and therefore this method is not feasible on a large scale. The microwave oven may, however, still be of interest in the laboratory as a screening method to analyze the composition of feedstock as well as to determine a range of optimal conditions for steam pretreatment.

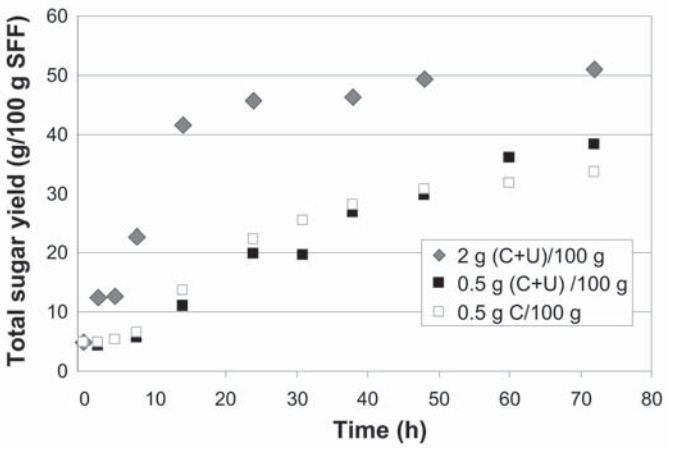

Fig. 5. Effect of enzyme loading in enzymatic hydrolysis of material after steam pretreatment at 190°C for 10 min.

Enzyme Loading

The effect of reducing the enzyme loading was also evaluated. Enzymatic hydrolysis after microwave pretreatment was performed previously both with and without the addition of Ultraflo to Celluclast. Only a small increase in total sugar yield (4%) was observed when using a mixture of 2 g of Celluclast + Ultraflo instead of 2 g of Celluclast/100 g of SFF slurry (5% DM content) following microwave pretreatment at 170°C for 40 min and 180°C for 30 min (results not shown).

To investigate further the effect of enzyme loading, three materials pretreated at different degrees of severity (i.e., 220°C for 5 min, 190°C for 10 min, and 170°C for 15 min) were selected for enzymatic hydrolysis with dosages of 0.5 g of Celluclast + Ultraflo mixture/100 g of SFF slurry and 0.5 g of Celluclast/100 g of SFF slurry. Figure 5 shows the maximum total sugar yield obtained at these enzyme loadings for the material pretreated at 190°C for 10 min. The yield obtained with 2 g of Celluclast + Ultraflo/100 g of SFF is also shown for comparison.

When 0.5 g of Celluclast + Ultraflo/100 g of SFF was used, a total yield of 38.4 g of sugars/100 g of SFF, corresponding to 55% of the theoretical, was obtained. When only Celluclast was used the total sugar yield was reduced to 33.5 g/100 g of SFF. The materials pretreated at 220°C for 5 min and at 170°C for 15 min showed curves similar to those presented in Fig. 5 although the yields were slightly lower than those obtained with the material pretreated at 190°C for 10 min.

Direct enzymatic hydrolysis with the supplementation of Ultraflo to the Celluclast resulted in a significant increase in yield compared with when Celluclast alone was used (Fig. 6). The maximum sugar yield for direct enzymatic hydrolysis, 36.6 g/100 g, corresponding to 52% of the

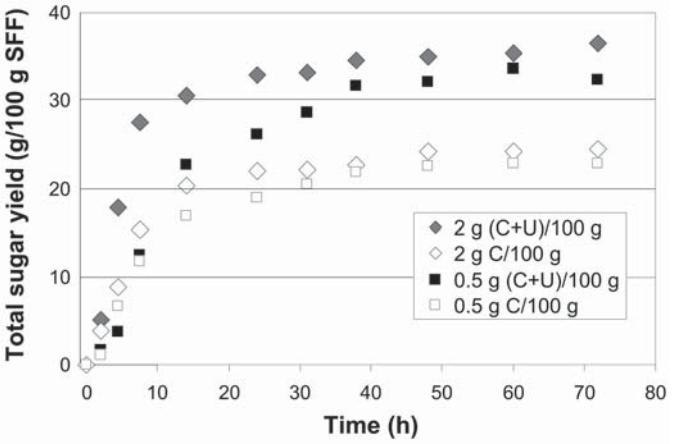

Fig. 6. Total sugar yield as function of time for direct enzymatic hydrolysis with enzyme loadings of 0.5 g of Celluclast + Ultraflo/100 g of SFF slurry, 0.5 g of Celluclast/100 g of SFF, 2 g of Celluclast + Ultraflo/100 g of SFF slurry, and 2 g of Celluclast/100 g of SFF.

theoretical, was achieved with 2 g of Cellulcast + Ultraflo mixture/100 g of slurry. When only Celluclast was used at the same enzyme loading, the yield decreased to 35.8% of the theoretical.

On the other hand, no significant difference in total sugar yield was observed between the 0.5- and 2-g loading for either two- or one-enzyme preparations, but the conversion rate was faster when the higher loading was used.

By contrast, a much less pronounced difference was observed between one- and two-enzyme preparations when enzymatic hydrolysis was preceded by pretreatment. Table 4 presents the yields of individual and total sugars obtained after direct enzymatic hydrolysis for 72 h, and after combined pretreatment and enzymatic hydrolysis with the different enzyme loadings.

At the lowest enzyme loading, pretreatment prior to enzymatic hydrolysis had a significant effect only on the glucose yield, since most of the pentosan could be readily hydrolyzed by direct enzymatic hydrolysis.

Conclusion

Wheat SFF, consisting of about 70 wt% carbohydrates on a DM basis, proved to be a suitable substrate to release sugars for ethanol production. If all the sugars in the SFF could be utilized, this would correspond to more than 25% of the glucose available in the starch fraction, which is used for ethanol production today. Pretreatment of the SFF could thus substantially improve the ethanol yield. Steam pretreatment at the optimal conditions,

Table 4
Individual and Total Sugar Yields Following Direct Enzymatic Hydrolysis of SFF and Following Combined Steam Pretreatment at 190°C for 10 min and Enzymatic Hydrolysis.

	Arabinose	Xylose	Glucose	Total
SP: 190°C for 10 min followed by EH				
2 g (C + U)/100 g SFF	13.4	21.2	17.2	51.8
0.5 g (C + U)/100 g SFF	8.8	15.5	14.1	38.4
0.5 g C/100 g SFF	6.6	12.5	14.4	33.5
Direct EH				
2 g (C + U)/100 g SFF	9.0	17.6	10.0	36.6
2 g C/100 g SFF	4.0	11.3	9.2	24.5
0.5 g (C + U)/100 g SFF	8.1	15.9	8.4	32.4
0.5 g C/100 g SFF	4.2	10.4	8.3	22.9

[a]SP, steam pretreatment; EH, enzymatic hydrolysis; C, celluclast; U, ultraflo.

190°C for 10 min, followed by enzymatic hydrolysis with 2 g of Celluclast + Ultraflo resulted in a total sugar yield of 74% of the theoretical. When a loading of 0.5 g of Celluclast + Ultraflo/100 g of SFF slurry was used, the total sugar yield decreased to 55% of the theoretical. Nevertheless, this yield is slightly higher than the overall total sugar yield obtained by direct enzymatic hydrolysis with 2 g of Celluclast + Ultraflo/100 g of SFF slurry, which resulted in 36.5 g/100 g of SSF, corresponding to 52% of the theoretical yield. Thus, pretreatment prior to enzymatic hydrolysis could reduce the enzyme loading without a reduction in the sugar yield.

Acid hydrolysis with 0.2% H_2SO_4 at 160°C for 20 min followed by enzymatic hydrolysis resulted in 88% of the theoretical yield. Because some acid is already used in the process to adjust the pH before fermentation, the addition of small amounts of acid in a pretreatment step could be a possibility.

A higher increase in sugars was obtained with acid hydrolysis using 1% H_2SO_4 at 130°C for 40 min followed by enzymatic hydrolysis, which resulted in approx 70 g/100 g of SFF. Because the addition of acid presents several drawbacks, such as the requirement of corrosion-resistant construction materials and a need for neutralization prior to fermentation, this higher acid concentration may not be feasible *(2)*.

To evaluate the different hydrolysis options, a study of economic feasibility, considering the possible increase in ethanol yield and the overall process cost, must be performed.

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