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Comparison of the Fermentability of Enzymatic Hydrolyzates of Sugarcane Bagasse Pretreated by Steam Explosion Using Different Impregnating Agents

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Abstract

Sugarcane bagasse is a potential lignocellulosic feedstock for ethanol production, since it is cheap, readily available, and has a high carbohydrate content. In this work, bagasse was subjected to steam explosion pretreatment with different impregnation conditions. Three parallel pretreatments were carried out, one without any impregnation, a second with sulfur dioxide, and a third with sulfuric acid as the impregnating agent. The pretreatments were performed at 205°C for 10 min. The pretreated material was then hydrolyzed using cellulolytic enzymes. The chemical composition of the hydrolyzates was analyzed. The highest yields of xylose (16.2 g/100 g dry bagasse), arabinose (1.5 g/100 g), and total sugar (52.9 g/100 g) were obtained in the hydrolysis of the SO_2-impregnated bagasse. The H_2SO_4-impregnated bagasse gave the highest glucose yield (35.9 g/100 g) but the lowest total sugar yield (42.3 g/100 g) among the three methods. The low total sugar yield from the H_2SO_4-impregnated bagasse was largely due to by-product formation, as the dehydration of xylose to furfural. Sulfuric acid impregnation led to a three-fold increase in the concentration of the fermentation inhibitors furfural and 5-hydroxymethylfurfural (HMF) and a two-fold increase in the concentration of inhibitory aliphatic acids (formic, acetic, and levulinic acids)

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compared to the other two pretreatment methods. The total content of phenolic compounds was not strongly affected by the different pretreatment methods, but the quantities of separate phenolic compounds were widely different in the hydrolyzate from the H_2SO_4-impregnated bagasse compared with the other two hydrolyzates. No major differences in the content of inhibitors were observed in the hydrolyzates obtained from SO_2-impregnated and non-impregnated bagasse. The fermentability of all three hydrolyzates was tested with a xylose-utilizing *Saccharomyces cerevisiae* strain with and without nutrient supplementation. The hydrolyzates of SO_2-impregnated and nonimpregnated bagasse showed similar fermentability, whereas the hydrolyzate of H_2SO_4-impregnated bagasse fermented considerably poorer.

Index Entries: Sugarcane bagasse; ethanol; pretreatment; *S. cerevisiae*

Introduction

The concern for climate change phenomena has focused scientific attention on the use of ethanol as a substitute or an additive to gasoline as transportation fuel. Lignocellulose is the most abundant renewable resource available for industrial production of fuel ethanol. Sugarcane bagasse represents the main lignocellulosic material to be considered in many tropical countries, since it is readily available at the sugar factory site at no additional cost and has high carbohydrate and low lignin content.

The cellulose in lignocellulosic materials should be hydrolyzed to glucose in order to give rise to a substrate amenable for ethanolic fermentation by yeasts or bacteria. Chemical and enzymatic methods are the most common techniques for hydrolyzing cellulose. In the chemical method, the hydrolysis is catalyzed by an acid, whereas in the enzymatic method cellulolytic enzymes act as catalysts.

An important advantage of the enzymatic hydrolysis, compared to the acid hydrolysis, is the low formation of by-products, which decrease the sugar yield and also diminish the ethanol formation rate during the fermentation of the hydrolyzate *(1)*. However, lignocellulosic materials should be pretreated prior to the enzymatic hydrolysis in order to make the cellulose accessible to the cellulolytic enzymes. Several pretreatment methods have been described for bagasse *(2–4)*. Steam explosion is a very efficient pretreatment method that has been studied for different lignocellulosic materials *(5–7)* including bagasse *(4,8,9)*. Although steam pretreatment has clear advantages over other pretreatment methods, it still results in the formation of by-products that inhibit the fermentation *(10,11)*. The toxicity of fermentation inhibitors in lignocellulose hydrolyzates, for example, aliphatic acids, furaldehydes, and phenolic compounds, has been studied thoroughly *(12–15)*. The strategy for dealing with fermentation inhibitors is a key question in the design of an economically feasible process for converting lignocellulose to ethanol *(16–18)*.

The formation of sugars and by-products during steam pretreatment and enzymatic hydrolysis of sugarcane bagasse and the fermentability of

the hydrolyzates have recently been studied *(19)*. It was found that hydrolyzates produced by pretreatment at 205°C during 10 min had the maximum sugar yield and gave the highest ethanol concentrations among the different steam pretreatment conditions investigated. However, the high fiber content of the residue after hydrolysis suggested that not all of the cellulose was hydrolyzed. Therefore, impregnation of bagasse with catalysts, such as sulfur dioxide or sulfuric acid, prior to steam pretreatment might be necessary. Steam pretreatment of bagasse impregnated with sulfuric acid has been performed previously *(8,9)*, whereas impregnation of bagasse with sulfur dioxide has to our knowledge not previously been reported.

The aim of the present work was to compare the sugar yields, by-product formation and fermentability of enzymatic hydrolyzates of sugarcane bagasse pretreated by steam explosion (1) without impregnation, (2) after impregnation with sulfur dioxide, and (3) after impregnation with sulfuric acid.

Materials and Methods

Raw Material

Fresh bagasse was kindly provided by the sugar mill "Granma" (Matanzas, Cuba) and transported to Lund University, Sweden, by air. Bagasse was fractionated to a particle size between 2.2 and 10 mm. The composition was analyzed by the Hägglund method for raw material analysis *(20)*.

Pretreatment

Pretreatment of bagasse was performed in the equipment previously described *(11)*. Bagasse corresponding to 300 g dry matter (DM) content was loaded into the preheated reactor. Steam was supplied from a boiler and the material was heated up to 205°C during 10 min. Then, the pretreated material was discharged into a cyclone connected to the outlet of the reactor. The pretreated material was collected and samples from the supernatant were analyzed to determine the yield of sugars and by-products. The fibrous material in the sample withdrawn for analyses was washed thoroughly with water to remove all soluble substances, dried at 105°C overnight and the fiber yield was determined gravimetrically.

In the experiment with sulfur dioxide impregnation, bagasse was placed in a plastic bag and SO_2 (1.1% w/w DM) was supplied from a gas cylinder. The amount of SO_2 added to the bag was estimated by weighing the cylinder. The absorbed amount of SO_2 was determined by weighing the bag before and after SO_2 addition. After 15 min at room temperature, the impregnated material was steam pretreated at 205°C for 10 min *(21)*.

For sulfuric acid impregnation, bagasse was mixed, in a plastic bag, with 150 mL dilute H_2SO_4, giving a final concentration of 1 g H_2SO_4/100 g DM, and stored at room temperature overnight. The impregnated material was steam pretreated afterwards at 205°C for 10 min *(21)*.

Enzymatic Hydrolysis

Pretreated bagasse was hydrolyzed by the cellulase mixture Celluclast 2L and the β-glycosidase preparation Novozym 188 (Novo Industri A/S, Bagsværd, Denmark). The activity of the Celluclast 2L was 90 FPU/g *(22)*. The β-glucosidase activity in Celluclast was 12 IU/g and in Novozym it was 390 IU/g *(23)*. The bagasse slurry was diluted with water, adjusted to pH 4.8 and mixed with 2.3 g Celluclast and 0.5 g Novozym. Water was added to a final weight of 500 g and the dry matter was 5% (w/w). Hydrolysis was allowed to proceed for 96 h at 40°C and with stirring. The pH was maintained at 4.8 by addition of 5 *M* NaOH. The hydrolyzate was subsequently separated by vacuum filtration. Samples of the hydrolyzate were taken for analysis of the chemical composition.

Microorganism and Growth Conditions

The recombinant *Saccharomyces cerevisiae* strain TMB 3001 was used. The TMB 3001 strain is a CEN.PK-derivative that expresses xylose reductase and xylitol dehydrogenase from the chromosomally integrated *Pichia stipitis* genes *XYL1* and *XYL2*, respectively, and over-expresses the homologous *XKS1* gene encoding xylulokinase *(24)*. The strains were maintained on YPD-agar plates containing 10 g/L yeast extract (Merck, Germany), 20 g/L peptone (Difco, Detroit, MI, USA), 20 g/L glucose (BDH, UK), and 20 g/L agar–agar (Merck, Germany). Precultures, inoculated from agar plates, were grown in 100 ml mineral medium *(25)* at 30°C overnight in 250-mL baffled Erlenmeyer flasks with agitation (150 rpm) in an orbital incubator (Gallenkamp, Leicester, UK). The cells were harvested in exponential phase by centrifugation (J-25I, Beckman, Fullerton, CA) at 1200*g* for 10 min and washed in 0.9% (w/v) NaCl.

Fermentation

Fermentations were run either with supplementation of the hydrolyzates with nutrients to final concentrations of 1 g/L yeast extract, 0.5 g/L $(NH_4)_2HPO_4$, 0.025 g/L $MgSO_4 \cdot 7H_2O$, and 1.38 g/L NaH_2PO_4 as previously described *(19)* or without nutrient supplementation. The pH of the hydrolyzates was adjusted to 5.5 with 2 *M* NaOH and the hydrolyzates were filter sterilized. The fermentations were performed under oxygen-limited conditions, in 50-mL fermentors with a working volume of 45 mL and sealed with rubber stoppers. Fermentors were equipped with cannulas for carbon dioxide removal and sampling. Fermentors were inoculated to a cell concentration of approx 0.2 g/L (DW). The cells were incubated at 30°C with magnetic stirring and fermentations were run for 48 h under aseptic conditions. Fermentations of solutions containing D-glucose (BDH Laboratory Supplies, Poole, England), D-xylose (Acros Organics, NJ), and nutrients in the same concentration as in the hydrolyzate were performed as references to determine ethanol productivity and yield in the absence of inhibitors.

Analyses

The samples from pretreatment, hydrolysis and fermentation were analysed by high performance liquid chromatography (HPLC). All samples were filtered through a 0.20 µm filter and diluted prior to HPLC analysis. The concentrations of cellobiose, glucose, xylose, and arabinose in the liquid collected after pretreatment and in the hydrolyzates were determined by using an HPLC system (Shimadzu LC-10AT, Kyoto, Japan) equipped with an RI-detector (Shimadzu RID-6A, Kyoto, Japan) and an HPX-87P column (Bio-Rad, Hercules, CA) operating at 80°C with degassed ultrapure water (Millipore, Bedford, MA) as the mobile phase at a flow rate of 0.5 mL/min. The concentrations of acetic, formic, and levulinic acids in the hydrolyzates were analyzed using an Aminex HPX-87H column (Bio-Rad) operating at 45°C with 5 m*M* H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min and detected with an RI-detector (Shimadzu RID-6A). HPLC results were processed using the EZChrom software (Scientific Software, Inc., San Ramon, CA).

Fermentation samples were analyzed on a Gilson HPLC (Gilson, Middletown, WI) using the Unipoint software (Gilson). Glucose, xylose, ethanol, xylitol, acetic acid, formic acid, and levulinic acid in fermentation samples were separated on an Aminex HPX-87H column (Bio-Rad) operating at 45°C with 5 m*M* H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min and detected with an RI-detector (Shimadzu RID-6A).

Furfural, HMF, and furfuryl alcohol in hydrolyzates and fermentation samples were analyzed with an HPLC system (Waters, Milford, MA) equipped with a BioSil C18 column (Bio-Rad) using a UV-detector (Waters 2487 Dual Absorbance Detector) set on a wavelength of 230 nm and operating at room temperature. The mobile phase consisted of 40% (v/v) aqueous methanol (BDH), adjusted to pH 3 with concentrated HCl and supplied at a flow rate of 0.6 mL/min. The results were processed using the EZChrom software.

Separate phenolic compounds were identified by HPLC and GC-MS. The HPLC analysis was performed using the same procedure as described for furanic compounds, except for the wavelength that was set to 254 nm. For GC-MS analysis, the phenolic compounds were extracted from the acidified hydrolyzates with ethyl acetate. The extracts were concentrated by evaporation of the ethyl acetate (Büchi Rotavapor-R, Glassapparatefabrik, Flawil, Switzerland), dried over sodium sulfate, silylated using BSTFA [bis (trimethylsilyl)-trifluoroacetamide] with traces of pyridine, separated by GC (Hewlett Packard 5890) on a 30 m, 0.32-mm-ID CP-SIL8 CB column (Chrompac, Middelburg, The Netherlands) using a temperature program from 80 to 280°C at 5°C/min and identified by MS (MS engine, 5989B, Hewlett Packard).

The total content of phenolic compounds was quantified colorimetrically by the Folin–Ciocalteau method *(26)*, using a U-1100 spectrophotometer (Hitachi Ltd., Tokyo, Japan). Vanillin was used as the calibration standard.

The biomass content was analyzed gravimetrically (as DW) at the beginning and at the end of the fermentations. Five-milliliter samples were vacuum filtered through 0.45 μm preweighed filters (Gelman Sciences, Ann Arbour, MI). The filtered cells were then washed with 15 mL ultrapure water (Millipore) and dried in a microwave oven (Whirlpool, MI) during 8 min at the power of 3.5 and weighed. OD measurements of the inoculum were performed using the U-1100 spectrophotometer (Hitachi Ltd.).

Calculations

The ethanol yield (g/g) from glucose was calculated as the amount of ethanol formed per gram of initial glucose. The ethanol yield from the sum of initial glucose and xylose was also calculated, and it is hereafter referred to as ethanol yield from total sugar. The maximum volumetric productivity [g/(l h)] was based on grams of ethanol produced per liter of culture medium per hour during the first 12 h of the fermentation. The maximum volumetric productivity in the reference was obtained after 6 h, but this was not useful for calculation of productivity in the hydrolyzates, since no ethanol was produced within this period. Therefore, the calculation of the productivities was based on the ethanol produced after 12 h, the second highest productivity for the reference. The maximum specific productivity, based on the respective volumetric productivities divided by the initial cell dry weight, was also calculated. The anaerobic growth yield (hereafter referred to as the biomass yield) was calculated as the increase in cell mass after 48 h divided by the initial glucose concentration.

Results

Pretreatment and Enzymatic Hydrolysis

The glucose yield after pretreatment was much higher with sulfuric acid impregnation than with SO_2 impregnation or without any impregnation (Table 1). Roughly 50% of the glucose content in the raw material was released already in the pretreatment step of the H_2SO_4-impregnated bagasse, while only one third was released during the enzymatic hydrolysis (Table 2). In contrast, only a negligible part of the glucose was released in the pretreatment stage after impregnation with sulfur dioxide and without any impregnation (Table 1).

The yields of xylose and arabinose after pretreatment with SO_2 impregnation and without any impregnation were considerably higher than what was obtained with the H_2SO_4 impregnation (Table 1). Both the xylose and the arabinose yield were higher with SO_2 impregnation than without any impregnation.

The fiber yield of the H_2SO_4-impregnated bagasse was more than 10% lower than in the samples subjected to the other two pretreatment conditions (Table 1). The SO_2-impregnated bagasse showed slightly lower fiber yield than the non-impregnated bagasse.

Table 1
Yields after Pretreatment, g/100 g Dry Bagasse (DB)[a]

Impregnating agent	Glucose yield	Xylose yield	Arabinose yield	Acetic acid yield	Fiber yield
None	1.0 (0.07)	6.1 (0.00)	0.6 (0.07)	3.5 (0.21)	72.1 (1.48)
SO_2	1.0 (0.14)	8.2 (0.14)	0.9 (0.07)	4.3 (0.14)	69.2 (0.92)
H_2SO_4	22.6 (0.84)	3.6 (0.42)	0.4 (0.14)	7.0 (0.42)	58.2 (1.77)

[a]Mean values from two replicates are indicated. The standard deviation is shown in parentheses.

Table 2
Sugar Yield After Pretreatment and Enzymatic Hydrolysis, g/100 g DB[a]

Impregnating agent	Glucose	Xylose	Arabinose	Total sugar
None	33.0 (0.42)	13.1 (0.07)	1.3 (0.14)	47.4
SO_2	35.2 (0.56)	16.2 (0.61)	1.5 (0.20)	52.9
H_2SO_4	35.9 (0.93)	5.9 (0.18)	0.5 (0.15)	42.3

[a]Mean values for at least three replicates. The standard deviation is shown in parentheses.

The highest glucose yield after pretreatment and enzymatic hydrolysis, 35.9 g/100 g dry bagasse (DB), corresponding to more than 80% of the theoretical yield, was reached in the hydrolysis of the H_2SO_4-impregnated bagasse (Table 2). The highest yields of xylose, arabinose, and total sugar were obtained in the hydrolysis of the SO_2-impregnated bagasse. The xylose obtained in the hydrolysis of the SO_2-impregnated bagasse was approx 61% of the theoretical yield, while the hydrolysis of the non-impregnated bagasse reached 50% and the H_2SO_4-impregnated only 22% of the theoretical yield. A considerable amount (39–54%) of the xylose in the hydrolyzates was formed during the enzymatic hydrolysis.

The yields of most of the fermentation-inhibiting compounds were much higher for the hydrolyzate obtained with H_2SO_4-pretreated bagasse than for the other hydrolyzates (Table 3). However, the yields of acetic acid and total phenolic compounds were rather similar for all three methods. With the H_2SO_4-method, all the acetic acid was released at the pretreatment stage, whereas only around 57 and 68% was released at this stage without impregnation and with SO_2 impregnation, respectively (Tables 1 and 3). Levulinic acid was detected only in the sample prepared by H_2SO_4 impregnation.

Determination of the concentration of total phenolics with the Folin–Ciocalteau method showed only a small variation, between 2.8 and 3.2 g/L, for the different hydrolyzates (Table 4). Thirteen different phenolic compounds were identified and quantified by GC-MS (Table 5, Fig. 1).

Table 3
Yields of Inhibitory By-products After Pretreatment and Enzymatic Hydrolysis, g/100 g DB[a]

Impregnating agent	Furfural	HMF	Formic acid	Acetic acid	Levulinic acid	Phenolic compounds
None	1.6 (0.00)	0.7 (0.00)	1.5 (0.07)	6.1 (0.21)	0.0 (0.00)	4.5 (0.14)
SO_2	1.6 (0.10)	0.7 (0.06)	1.5 (0.00)	6.3 (0.26)	0.0 (0.00)	4.4 (0.21)
H_2SO_4	4.4 (0.31)	1.8 (0.17)	3.2 (0.14)	6.5 (0.35)	3.6 (0.15)	4.0 (0.08)

[a]Mean values for at least three replicates are indicated. The standard deviation is shown in parentheses.

Table 4
Composition of the Hydrolyzates, g/L[a]

Component	NI	SD	SA
Glucose	20.6 (0.14)	22.8 (0.63)	25.6 (1.20)
Xylose	8.1 (0.01)	10.6 (0.21)	4.4 (0.06)
Arabinose	0.8 (0.14)	0.9 (0.20)	0.4 (0.13)
Furfural	1.1 (0)	1.2 (0.01)	3.1 (0.26)
HMF	0.4 (0)	0.4 (0)	1.4 (0.14)
Acetic acid	4.2 (0.01)	4.4 (0.11)	4.9 (0.23)
Formic acid	1.0 (0)	1.1 (0.05)	2.5 (0.11)
Levulinic acid	0 (0)	0 (0)	2.7 (0.20)
Phenolic compounds	3.2 (0)	3.1 (0.01)	2.8 (0.09)

[a]NI, pretreatment with no impregnation; SD, pretreatment by impregnation with SO_2; SA, pretreatment with impregnation with H_2SO_4. Mean values from at least three replicates. Standard deviation is shown in parentheses.

Although it was possible to identify the same phenolic compounds in all three hydrolyzates, the concentration of the separate phenols was very different in different hydrolyzates (Table 5). The results showed no major differences in the formation of aromatic compounds between the hydrolyzates of SO_2-impregnated and nonimpregnated bagasse. The difference between these two hydrolyzates and the H_2SO_4-impregnated bagasse hydrolyzate was, however, apparent. The concentrations of phenol and guaiacol were 4–7 times higher in the H_2SO_4-impregnated bagasse hydrolyzate than in the other hydrolyzates. The concentrations of benzoic and vanillic acid were approx two times higher in the H_2SO_4-impregnated bagasse hydrolyzate than in the other hydrolyzates. The concentrations of *p*-coumaric acid, caffeic acid, and ferulic acid were 2–9 times lower in the H_2SO_4-impregnated bagasse hydrolyzate than in the other hydrolyzates.

The lignin content of the solid residue after hydrolysis of the H_2SO_4-impregnated bagasse was almost 90 g/100 g DB (Table 6). Lignin contents

Table 5
Aromatic Compounds Identified in the Hydrolyzates[a]

Compound	Concentration (mg/L)		
	NI	SD	SA
Phenol	3	3	22
Catechol	17	10	14
Guaiacol	4	3	16
4-Hydroxybenzaldehyde	110	100	100
Vanillin	50	50	58
Syringaldehyde	26	25	35
Benzoic acid	5	5	8
4-Hydroxybenzoic acid	11	9	10
Protocatechuic acid	5	3	3
Vanillic acid	20	15	36
p-Coumaric acid	480	410	170
Caffeic acid	9	5	1
Ferulic acid	210	190	56

[a]NI, pretreatment with no impregnation; SD, pretreatment with impregnation with SO_2; SA, pretreatment with impregnation with H_2SO_4.

Table 6
Composition of the Raw Bagasse and the Hydrolysis Residues in g/100 g DB[a]

Component	Raw bagasse	NI residue	SD residue	SA residue
Lignin	24.4 (0)	60.4 (0.05)	71.6 (0.12)	87.7 (0.18)
Glucan	44.7 (1.13)	13.5 (0.31)	10.9 (0.49)	1.8 (0.15)
Xylan	26.4 (0.24)	5.9 (0.20)	4.3 (0.09)	0 (0)
Arabinan	2.9 (0.15)	0.6 (0.01)	0 (0)	0 (0)

[a]Determined using the Hägglund method (20). Mean values from two replicates are indicated. The standard deviation is shown in parentheses.

of around 60 and 70 g/100 g DB were achieved in the residues of the nonimpregnated and SO_2-impregnated bagasse. The hydrolysis residues contained approximately 20, 15, and 2 g residual polysaccharides per 100 g DB in nonimpregnated, SO_2-impregnated, and H_2SO_4-impregnated bagasse, respectively. Glucan was the only polysaccharide found in the solid residue of H_2SO_4-impregnated bagasse. Xylan was found in the solid residues of SO_2-impregnated and nonimpregnated bagasse, while arabinan was detected only in the residue of the nonimpregnated bagasse.

Fermentability

Under the conditions used, the hydrolyzates produced by pretreatment without impregnation (hydrolyzate NI) and with impregnation with sulfur dioxide (hydrolyzate SD) were found to be fermentable within 48 h, but the hydrolyzates prepared by pretreatment with impregnation with

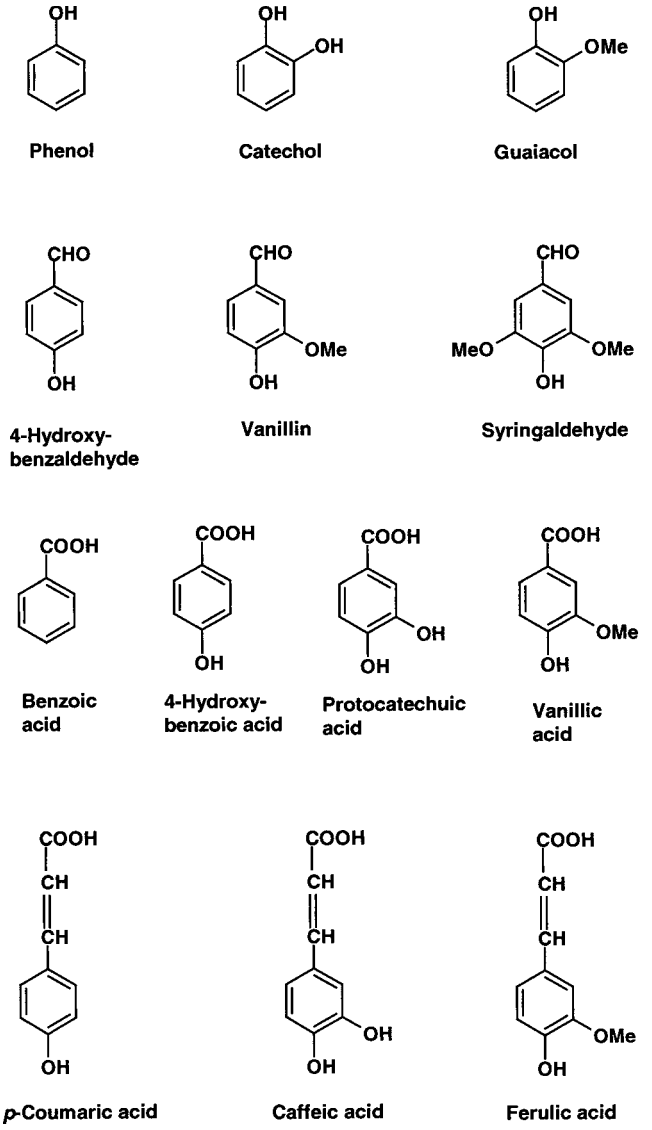

Fig. 1. Aromatic compounds detected in the hydrolyzates by GC-MS analysis.

sulfuric acid (hydrolyzate SA) would not ferment (Fig. 2, Table 7). No major differences between the fermentability of the hydrolyzates NI and SD were detected. A lag in the ethanol production during the first 6 h of fermentation was observed (Fig. 2). The productivity, the yield, and the ethanol concentration increased during the course of the fermentation and the ethanol concentration and the ethanol yield after 48 h even reached values comparable with those of the reference. The biomass yield was considerably lower in the hydrolyzates than in the reference even after 48 h (Table 7). No growth was observed in the hydrolyzates of H_2SO_4-impregnated bagasse.

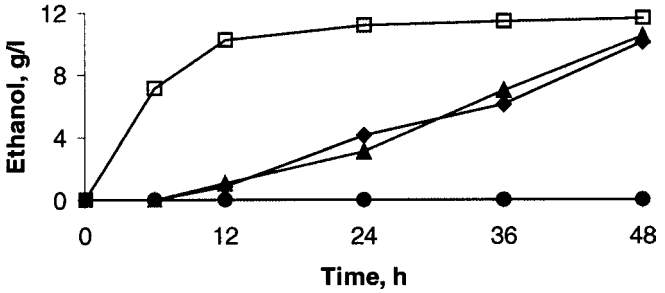

Fig 2. Ethanol production by *S. cerevisiae* from hydrolyzates obtained after pretreatment with no impregnation (◆), SO_2 impregnation (▲), and H_2SO_4 impregnation (●) and from the reference solution (□).

Table 7
Fermentability of the Hydrolyzates from Nonimpregnated (NI), SO_2-Impregnated (SD) and H_2SO_4-Impregnated (SA) Bagasse[a]

Medium	Supplement	$Y^{24}_{E/G}$ (g/g)	$Y^{24}_{E/TS}$ (g/g)	$Y^{48}_{E/G}$ (g/g)	$Y^{48}_{E/TS}$ (g/g)	Q_{max} [g/(L·h)]	q_{max} [g/(L·h)]	$Y_{X/G}$ (g/g)
Hydrolyzate	YE	0.20	0.14	0.49	0.34	0.08	0.40	0.029
NI	None	0.11	0.07	0.49	0.34	0.04	0.20	0.019
Hydrolyzate	YE	0.14	0.09	0.45	0.31	0.08	0.40	0.034
SD	None	0.11	0.07	0.44	0.31	0.05	0.25	0.024
Hydrolyzate	YE	0	0	0	0	0	0	0
SA	None	0	0	0	0	0	0	0
Reference	—	0.48	0.33	0.50	0.35	0.85	4.25	0.071

[a]$Y^{24}_{E/G}$, ehtanol yield from glucose after 24 h; $Y^{24}_{E/TS}$, ethanol yield from total sugar after 24 h; $Y^{48}_{E/G}$, ethanol yield from glucose after 48 h; $Y^{48}_{E/TS}$, ethanol yield from total sugar after 48 h; Q_{max}, maximum volumetric productivity (Q^{12}_{max} for hydrolyzates and Q^{6}_{max} for reference); q_{max}, maximum specific productivity (q^{12}_{max} for hydrolyzates and q^{6}_{max} for reference); $Y_{X/G}$, biomass yield from glucose after 48 h.

After 24 h, around 25% of the initial glucose was consumed in hydrolyzates NI and SD, while xylose consumption could be observed only after 48 h (Table 8). Xylose consumption was roughly five times lower than in the reference. In hydrolyzate SA, only a minor consumption of glucose could be detected after 48 h, while xylose consumption did not occur at all.

The supplementation with nutrients was found to only slightly stimulate the ethanol production rate after 12 h of fermentation (Table 7). This stimulation was almost negligible compared to the reference, in which a very rapid fermentation took place. On the other hand, the addition of nutrients to hydrolyzate SA did not show any stimulating effect.

The concentrations of furaldehydes during fermentation of SD and SA hydrolyzates are shown in Fig. 3. The data from the fermentation of hydrolyzate NI were similar to the data obtained with hydrolyzate SD and are therefore not shown. As can be seen from Fig. 3A, all furfural was

Table 8
Consumed Sugar after 24 and 48 h of Fermentation of Hydrolyzates from Nonimpregnated (NI), SO_2-Impregnated (SD), and H_2SO_4-Impregnated (SA) Bagasse

		Glucose (% consumed)		Xylose (% consumed)	
Medium	Supplement	24 h	48h	24 h	48 h
Hydrolyzate NI	YE	26	97	0	10
	None	23	100	0	8
Hydrolyzate SD	YE	28	100	0	11
	None	24	95	0	6
Hydrolyzate SA	YE	1	9	0	0
	None	2	2	0	0
Reference	—	100	100	35	53

converted in hydrolyzate SD during the first 24 h. Furfural conversion was slower than previously reported *(19)*. Except that the rate was a bit slower, HMF showed a similar conversion trend as furfural. Furfuryl alcohol was formed while furfural was converted. The sum of the concentrations of furfural and furfuryl alcohol was roughly constant during the entire course of the fermentation. The concentration of furfuryl alcohol reached a maximum simultaneously with the depletion of furfural and then it declined slowly, probably due to evaporation. In hydrolyzate SA, the conversion of furaldehydes was only marginal (Fig. 3B). The concentrations of aliphatic acids did not change to any greater extent during the course of the fermentations, either for hydrolyzates NI, SD, or SA (data not shown).

Discussion

Pretreatment and Hydrolysis

Three different approaches to perform pretreatment of sugarcane bagasse were compared with respect to yield of sugars, formation of fermentation inhibitors, and fermentability of the resulting hydrolyzates. The choice of impregnating agent, SO_2 or H_2SO_4, was shown to have a major impact on all these parameters.

The yields of glucose and xylose under different pretreatment conditions provided an indication of the efficiency of the conversion of cellulose and hemicellulose to the corresponding monosaccharides. The breakdown of hemicellulose was also reflected by the yield of acetic acid, which is formed mainly by the hydrolysis of acetylated β-D-xylopyranose residues in lignocellulose.

The high glucose yield obtained after the pretreatment of the H_2SO_4-impregnated bagasse indicates that most of the cellulose was already

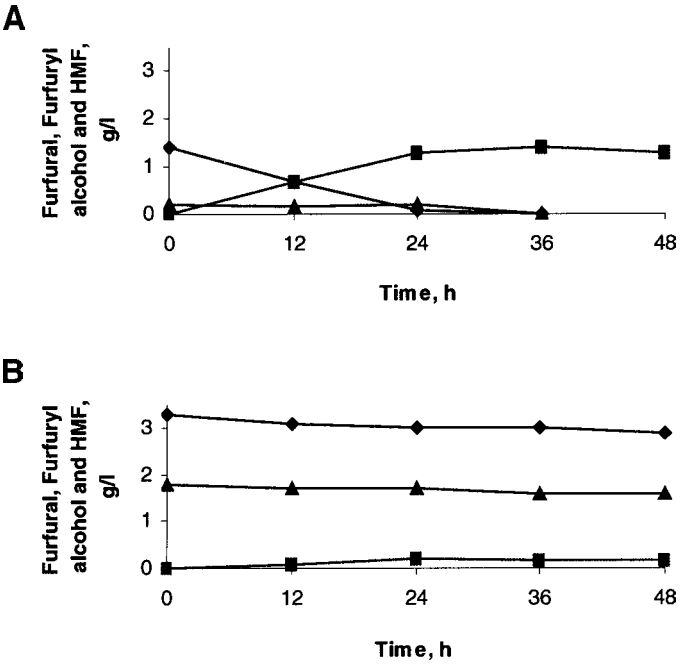

Fig. 3. Conversion of furfural(◆) and HMF (▲) and formation of furfuryl alcohol (■) during fermentation of unsupplemented hydrolyzate SD **(A)** and hydrolyzate SA **(B)**.

hydrolyzed at the pretreatment stage. A considerable hydrolysis of hemicellulose also took place at the pretreatment stage as can be seen by the high concentration of acetic acid (Table 1). The very low content of polysaccharides in the hydrolysis residue (Table 6) points to an almost complete conversion of cellulose and hemicellulose. However, the severe conditions of this pretreatment method in addition caused a high degree of degradation of the released sugars. This is indicated by the low yields of xylose and arabinose (Table 2), the high concentrations of furfural and HMF (Table 3), and the high concentrations of formic and levulinic acid (Table 3). Studies of spruce acid hydrolyzates indicated that if the severity was increased even further, a decrease in the concentrations of furaldehydes would occur, while the concentrations of aliphatic acids remained at a high level *(1)*.

That the yield of xylose was lower than the yield of glucose can be attributed to a higher degree of destruction of the former due to longer exposure to conditions resulting in dehydration and formation of the corresponding furaldehyde. Hemicellulose does not have the crystallinity of cellulose *(27)* and consequently is to a higher degree susceptible to hydrolysis. Since hemicellulose-derived sugars, such as xylose and arabinose, are formed at an earlier stage, they are thus exposed to the dehydrating agents for a longer time. That the concentration of furfural was higher than HMF supports this assumption. Furfural and HMF were, in turn, partially degraded even further, as indicated by the high yield of formic and levulinic acids (Table 3).

The substantially lower fiber yield after pretreatment of H_2SO_4-impregnated bagasse, compared to SO_2-impregnated and nonimpregnated bagasse, could be due to a higher degree of hydrolysis of cellulose and hemicellulose at the pretreatment stage. For the other two pretreatment methods, almost all cellulose and approximately half of the hemicellulose were hydrolyzed at the enzymatic hydrolysis stage. However, there were some minor differences between these two methods. The yields of xylose, arabinose, and acetic acid (Tables 1 and 2) show that pretreatment without impregnation led to a lower conversion of hemicellulose during the pretreatment stage compared with the enzymatic hydrolysis. Sulfur dioxide catalysis caused a slightly higher conversion of hemicellulose during the pretreatment stage, but also stimulated the cellulose saccharification during the enzymatic hydrolysis stage.

The catalyzing effect of sulfur dioxide on the hydrolysis of the bagasse polysaccharides was as expected weaker than that of sulfuric acid. However, it is also evident that impregnation with sulfur dioxide resulted in less degradation of sugars released in the pretreatment compared to impregnation with sulfuric acid. On the other hand, the formation of approximately similar amounts of by-products from bagasse impregnated with sulfur dioxide as from nonimpregnated bagasse implies that sulfur dioxide did not appreciably stimulate the degradation of sugars compared to the treatment without impregnation. Furthermore, it has been found earlier that the addition of SO_2 improved the recovery of hemicellulose sugars compared to pretreatment without impregnation (J. N. Saddler, personal communication).

Although the superior effect of sulfur dioxide was evident, a partial degradation of hemicellulose-derived sugars occurred nevertheless. This was probably due to the relatively high temperature and the long residence time. Therefore, less severe conditions than tested in this work could give higher sugar yields. Looking for optimum conditions for SO_2-catalyzed steam pretreatment of bagasse is a desirable future goal. There should be a potential for increasing the sugar yields if the steam pretreatment conditions would be optimized, since the overall sugar yield of 52.9 g/100 g DB obtained in this study (Table 2) was lower than the yields achieved under optimized steam pretreatment conditions with H_2SO_4 impregnation *(8,9)*.

The concentration of total phenolic compounds did not seem to be particularly affected by the impregnation method. It has previously been observed that formation of phenolic compounds during acid hydrolysis of spruce generally increases with increasing severity and reaches a maximum, after which the concentration remains essentially constant *(18)*. Possibly, the severity of the different pretreatments performed in this work was high enough to result in similar concentrations of phenols.

However, looking at the separate phenolic compounds quantified in the GC-MS analyses, there was a very clear difference between pretreatment with sulfuric acid, on one hand, and pretreatment with sulfur dioxide or no impregnating agent, on the other hand (Table 5). The product

pattern formation with respect to the aromatic compounds was very similar after sulfur dioxide impregnation and without any impregnating agent. In the sample from sulfuric acid impregnation, there was less of the phenylpropane-derivatives *p*-coumaric acid, caffeic acid, and ferulic acid, but more of the smaller aromatic compounds phenol and guaiacol. This indicates that the degradation of the aromatic components has been pushed further in the sulfuric-acid-impregnated material, even if this was not reflected by a higher content of total phenolics as determined with the Folin–Ciocalteau method.

Fermentability

Because a relatively small inoculum was used in this work, the fermentation rates and xylose utilization observed in the fermentable hydrolyzates were lower than those observed previously *(19)* using the same yeast strain. Two different trends in the fermentability of the hydrolyzates were clearly observed in this work. That difference should be connected to the composition of the hydrolyzates, particularly to the content of fermentation-inhibiting compounds.

Aliphatic acids, such as acetic, formic and levulinic acids, furaldehydes, such as furfural and HMF, and phenolic compounds are three well-known groups of fermentation inhibitors in lignocellulose hydrolyzates. Previous investigations have demonstrated the key role of phenolic compounds as inhibitors in hydrolyzates of wood *(12,13,18)* and bagasse *(11,19)*. In the hydrolyzates studied here, aliphatic acids and furaldehydes reached quite high levels and may be more important as fermentation inhibitors than for instance in some of the spruce hydrolyzates.

Although the concentration of acetic acid was higher in the hydrolyzate produced by impregnation with sulfuric acid than in the other hydrolyzates, it was quite high in all hydrolyzates (Table 4). Therefore, it is unlikely that acetic acid played the decisive role in causing the difference in fermentability that was observed. On the other hand, formic acid and levulinic acid may well be involved in causing the particularly poor fermentability of the hydrolyzate from H_2SO_4-impregnated bagasse, since the concentrations in that hydrolyzate were very much higher than in the other hydrolyzates. In addition, it is known that both formic acid and levulinic acid are more inhibitory to yeast metabolism than acetic acid *(1)*.

There is an apparent contradiction, since the individual concentration of each of these three aliphatic acids is lower than 100 m*M*, which could be regarded as stimulatory rather than inhibitory *(1,28)*. Furthermore, the toxicity of all three acids under the fermentation conditions used in this work could be affected by the fact that the fraction of undissociated acid was relatively low because the extracellular pH was higher than the pKa of all the three acids. However, the combined molar concentration of the aliphatic acids in the hydrolyzate prepared by H_2SO_4-catalyzed steam pretreatment was around 200 m*M*, whereas in the other hydrolyzates it was no more than 90 m*M*. Perhaps, this can explain the

difference in the fermentability of the hydrolyzates. It is probable that different aliphatic acids exert additive or even synergistic inhibitory effect.

Furaldehydes also seem to play an essential role in the strong inhibition of the fermentation of hydrolyzate SA. Furfural is a very well known inhibitor of CO_2 evolution *(29)*, ethanol formation rate, and specific growth rate *(30)*. The lower consumption rate of HMF could lead to accumulation during fermentation, thus contributing to longer inhibition effects than furfural *(1,31)*.

The hydrolyzates that fermented well were able to convert furfural and HMF, whereas in the unfermentable hydrolyzate there was not a clear conversion of the furaldehydes (Fig. 3). Furaldehydes are converted to non-inhibitory forms, such as furfuryl alcohol, which facilitates fermentation of the hydrolyzates *(32)*. The lower rates of furfural and HMF conversion compared with previous results *(19)* might be related to the lower yeast cell-mass concentration used in this work.

Previously, the potential of using a recombinant xylose-utilizing *S. cerevisiae* strain for fermenting enzymatic hydrolyzates of sugarcane bagasse was demonstrated *(19)*. However, those hydrolyzates were supplemented with small amounts of yeast extract and minerals. In the present work, some of the hydrolyzates were supplemented with the same nutrients and in the same amounts as previously. However, the improvement of the ethanol production rate by the addition of the nutrients was very limited. This fact shows that *S. cerevisiae* strain TMB 3001 can ferment enzymatic hydrolyzates of SO_2-impregnated and nonimpregnated bagasse even without nutrient supplementation. This corroborates the prospective of using this yeast strain for fuel ethanol production from bagasse. Nevertheless, since the inhibitors contained in bagasse hydrolyzates affect the fermentation rate and xylose utilization, adaptation of this strain to the inhibitors would be a desirable future achievement.

In this work, major differences in composition and fermentability of bagasse hydrolyzates produced using different impregnating agents were demonstrated. The impregnation method with sulfur dioxide was shown to improve the sugar yield without resulting in increased inhibitor formation. Impregnation with sulfuric acid resulted in a hydrolysate with poor fermentability. The concentrations of aliphatic acids and furaldehydes were higher in the hydrolysate from bagasse impregnated with sulfuric acid, and GC-MS analysis also revealed that the concentrations of separate phenolic compounds were very different compared with what was obtained with the two other pretreatment methods. Whether the difference in the composition of phenolics affects the fermentability is a question that deserves further attention as well as the mechanisms by which the choice of impregnating agent affects the by-product formation.

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