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Comparison of Different Methods for the Detoxification of Lignocellulose Hydrolyzates of Spruce

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Abstract

This study describes different detoxification methods to improve both cell growth and ethanol production by Baker's yeast, *Saccharomyces cerevisiae*. A dilute-acid hydrolyzate of spruce was used for the all detoxification methods tested. The changes in the concentrations of fermentable sugars and three groups of inhibitory compounds—aliphatic acids, furan derivatives, and phenolic compounds—were determined and the fermentability of the detoxified hydrolyzate was assayed. The applied detoxification methods included: treatment with alkali (sodium hydroxide or calcium hydroxide); treatment with sulfite (0.1% [w/v] or 1% [w/v] at pH 5.5 or 10); evaporation of 10% or 90% of the initial volume; anion exchange (at pH 5.5 or 10); enzymatic detoxification with the phenoloxidase laccase; and detoxification with the filamentous fungus *Trichoderma reesei*. Anion exchange at pH 5.5 or 10, treatment with laccase, treatment with calcium hydroxide, and treatment with *T. reesei* were the most efficient detoxification methods. Evaporation of 10% of the initial volume and treatment with 0.1% sulfite were the least efficient detoxification methods. Treatment with laccase was the only detoxification method that specifically removed only one group of the inhibitors, namely phenolic compounds. Anion exchange at pH 10 was the most efficient method for removing all three major groups of inhibitory compounds; however, it also resulted in loss of fermentable sugars.

Index Entries: Detoxification; inhibition; ethanol production; *S. cerevisiae*; softwood.

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Introduction

Owing to the need for environmentally sustainable energy sources, lignocellulose is considered an attractive raw material for the production of fuel ethanol *(1,2)*. Lignocellulose, such as forestry wastes, can be hydrolyzed to sugars by using, for example, dilute sulfuric acid. However, a major problem associated with dilute-acid hydrolysis of lignocellulosic biomass is the poor fermentability of the produced hydrolyzates *(3,4)*. A broad range of compounds is liberated and formed during hydrolysis, and some are toxic to the fermenting microorganism. Inhibitors of fermentation include: furan derivatives, such as furfural and 5-hydroxy-methylfurfural (5-HMF); aliphatic acids, such as acetic acid, formic acid, and levulinic acid; and phenolic compounds.

Furfural and 5-HMF are formed when monosaccharides (pentoses and hexoses, respectively) released during hydrolysis are degraded *(5)*. Furfural and 5-HMF can be further broken down to formic and levulinic acids. Acetic acid is liberated from hemicellulose, which is easily degraded *(6)*. A minor part of lignin is degraded during the hydrolysis process, generating a wide range of aromatic compounds *(6)*.

During fermentation, furfural is reduced by yeast cells to furfuryl alcohol *(7,8)*. Furfural creates a lag-phase in the ethanol formation, but does not reduce the final ethanol yield *(9)*. Furfural is consumed more rapidly than 5-HMF *(3)*. The inhibitory effect of weak acids is pH-dependent. The undissociated form of a weak acid permeates the cell membrane and dissociates in the cytosol *(10)*. The protons are removed by the plasma membrane ATPase in order to neutralize the decrease in internal pH *(11)*. This requires adenosine triphosphate (ATP), which under anaerobic conditions is achieved by increased ethanol formation *(12,13)*. Very high concentrations of weak acids may result in acidification of the cytoplasm *(14)* and cell death. The mechanism of inhibition by aromatic compounds has not yet been elucidated; however, low molecular-mass phenolics are inhibitory *(15–17)*. Clark and Mackie *(15)* identified several inhibitory phenolics in a pine hydrolyzate. Among the phenolic compounds was a group of lignin degradation products referred to as Hibbert's ketones. Compounds from all three major groups—aliphatic acids, furan derivatives, and phenolic compounds—contribute to the inhibition. In addition, interaction effects between compounds have been observed *(18)*. Chemical, physical, and biological methods can be used to remove inhibitors prior to the fermentation, which increases the fermentability *(1)*. These include pH adjustment to 10 with calcium hydroxide of a sugar-cane bagasse hydrolyzate followed by fermentation with *Pichia stipitis (19)*; pH adjustment to 10 with calcium, barium, sodium, and magnesium hydroxides of dilute-acid hydrolyzates; sulfite addition *(20,21)*, ion-exchange resins, and activated-charcoal treatment of pine hydrolyzates followed by fermentations with *Clostridium acetobutylicum (22)*; activated charcoal and sulfite treatment of dilute-acid hydrolyzate from eucalyptus wood

followed by fermentation by *Debaromyces hansenii (23)*; treatment of steam-pretreated willow hydrolyzate with the phenoloxidizing enzyme laccase from *Trametes versicolor (17)*; and treatment with the filamentous fungus *T. reesei (24)* followed by fermentation with *S. cerevisiae*. Detoxification increases the cost of the process *(2)*; hence, selecting a cheap but efficient detoxification step is important. Some detoxification methods, such as treatment with charcoal and calcium hydroxide, have been reported to cause sugar losses *(22,25)*, which would negatively affect the economy of the process.

However, the effect of the different methods referred to above cannot be compared with each other because different hydrolyzates and different microorganisms were used in the fermentations. In this study, wood chips from spruce were used as a model substrate for dilute-acid hydrolysis. The same hydrolyzate was then treated with selected detoxification methods and analyzed for changes in composition. The analyses were performed to characterize the chemical effect of the various detoxification methods on the three major groups of inhibitory compounds. The efficiency of each detoxification method was subsequently assayed in fermentations with Baker's yeast, *S. cerevisiae*.

Methods

Preparation of Hydrolyzates

The raw material was freshly chipped Norway spruce, *Picea abies*, with the following composition (% of dry weight, DW): glucan, 41.6; mannan, 11.5; xylan, 4.7; galactan, 2.0; arabinan, 1.1; lignin, 25.7; extractives, 5.4; and ash, 0.3% *(26)*. Hydrolysis was performed in a Masonite gun batch reactor (Rundvik, Sweden). The chips, which were less than 10 mm in size, were impregnated with dilute sulfuric acid to a final concentration of 0.5% (w/w liquid) and stored for 24 h in room temperature. The material was then charged into the reactor and treated with saturated steam at 222°C (23 bar) for 7 min (the heating-up time, 20 s, was excluded). The reaction was started by direct steam injection. When the pre-set time had elapsed, the material was discharged into a collecting vessel. The hydrolyzate was recovered after separation of solid and liquid fractions by filtration. The liquid fraction, hereafter referred to as the hydrolyzate, had a pH of 1.9.

Detoxification

Treatment with Alkali

Treatment with alkali was performed by adding 20% (w/v) $Ca(OH)_2$ or 10% (w/v) NaOH to pH 10. After 1 h, the hydrolyzate was filtered. The pH was then readjusted to 5.5 with H_2SO_4, after which the hydrolyzate was filtered.

Treatment with Sulfite

The pH was adjusted with NaOH, after which the sulfite was added. Two sulfite (Na_2SO_3) concentrations were used: 0.1 or 1% (w/v) at pH 5.5 or 10. The samples were kept under an atmosphere of helium for 1 h and then filtered. The pH of the samples initially adjusted to pH 10 were readjusted to pH 5.5 with H_2SO_4 and the samples were then refiltered.

Evaporation

Evaporation was performed in a Turbo Vap 500 (Zymarck, Hopkinton, MA). Two samples were prepared, in which 10 or 90% of the initial volume was removed. The concentration of nonvolatile compounds was restored by adding water to 100% of the initial volume. The pH was adjusted to 5.5 with NaOH (10% w/v).

Anion Exchange

Anion exchange resin (AG 1-X8, Bio-Rad, Richmond, VA) was added until pH 5.5 or 10 was obtained. The hydrolyzate was treated with the polystyrenedivinylbenzene-based anion-exchange resin for 1 h in a batch procedure.

Treatment with Laccase

Treatment with laccase was performed at pH 5.5 (adjusted with 10% NaOH (w/v) as described previously *[17]*). A control treatment was performed along with enzyme treatment by adding water instead of enzyme solution. The control was performed to account for any changes in the composition of the hydrolyzate occurring during the incubation owing to factors other than enzymatic activity.

Treatment with *Trichoderma reesei*

Trichoderma reesei RUT C30 NRRL 11460 was maintained and fungal fermentations were performed as described previously *(24)*, with the exception that glucose (BDH Chemicals, LTD, Poole, UK) was used to prepare fungal inoculum instead of Solka floc. The inoculum constituted 10% (v/v) of the final volume. A control treatment was performed along with the fungal fermentation by adding water instead of inoculum to account for any changes in the composition of the hydrolyzate during the treatment occurring owing to factors other than fungal growth. Both treatments were performed under aseptic conditions.

Analyses of Hydrolyzate Composition

After treatment with different detoxification methods, the hydrolyzate was analyzed for concentrations of sugars, furfural, 5-HMF, acetic acid, formic acid, levulinic acid, and phenolic compounds in general, and Hibbert's ketones in particular. Sugar concentrations were determined by high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) with a refractive index detector (RID-10A, Shimadzu, Kyoto, Japan). Cellobiose, xylose, galactose, arabinose, and mannose were separated on

an HPX-87P column (Bio-Rad, Hercules, CA), at 80°C, with ultra-pure water as the mobile phase at a flow rate of 0.5 mL/min. The system was equipped with a Carbo-P Refill Cartridge (Bio-Rad) prior to the HPX-87P column.

The concentrations of furfural and 5-HMF were determined by a Gynkotek HPLC system 480 equipped with a diode array detector (DAD) UVD 340S (Gynkotek, Germering, Germany). The samples were diluted in distilled water and directly injected on a C-18 column (Nucleosil 100-5 C18, Merck, Darmstadt, Germany) and eluted with a gradient of 5–100% (v/v) methanol and 0.025 % (v/v) of trifluoroacetic acid with a flow rate of 0.8 mL/min. The purity of the peaks was checked using the DAD and the amounts were determined using syringic acid in water as the internal standard.

The concentrations of formic, acetic, and levulinic acid were determined using a DX500 HPLC system (Dionex, Sunnyvale, CA) and an AS11HC column, eluted with 80% (v/v) water and 20% (v/v) of a mixture consisting of 0.4 m*M* NaOH and methanol (50% v/v) at a flow rate of 1.4 mL/min. The samples were directly injected after dilution with water and filtration through a 0.2-µm filter (MFS-25). The amounts of aliphatic acids were determined from external calibration curves using a conductivity detector.

The phenolic compounds were isolated from the aqueous hydrolyzates by solid-phase extraction. For each sample an Isolute ENV+ column (International Sorbent Technology, Mid Glamorgan, UK) was activated by methanol and distilled water. An aliquot, usually 3 mL, was passed through the column, which then was washed by water and dried with a stream of nitrogen. The sample was eluted by methanol and dichloromethane. An internal standard, 3,4-dimethoxybenzoic acid methylester in dichloromethane, was added. The samples were partly evaporated and redissolved in dichloromethane, dried with $MgSO_4$ and silylated with bistrismethylsilyltrifluoroacetamide (BSTFA) with traces of pyridine. The resulting trimethylsilyl (TMS)-derivatives were separated by gas chromatography (GC) (Hewlett Packard 5890) on a 30-m, 0.32-mm ID CP-SIL8 CB column (Chrompac, Middleburg, The Netherlands) using a temperature program from 80–280°C at 5°C/min. The phenolic character of the different peaks was confirmed by GC-MS (MS engine, 5989B, Hewlett Packard, Avondale, PA) and peaks for the five isomeric Hibbert's ketones were identified using reference compounds. The peak areas from the Hibbert's ketones and the other phenolic peaks with similar retention properties were integrated and summarized as the total phenolic content. The total amount of phenolics was also determined spectrophotometrically with the Prussian blue method *(27)*.

Fermentation

Compressed Baker's yeast, *S. cerevisiae* (Jästbolaget AB, Rotebro, Sweden), was used in all fermentations. Prior to the fermentation, the pH of the hydrolyzate was adjusted to 5.5 with sodium hydroxide (2.5 *M*). The hydro-

lyzate was supplemented 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. The fermentations of only 35 g/L glucose (BDH) and nutrients as aforementioned are hereafter referred to as the reference fermentations. All fermentations were carried out in 25-mL flasks containing 20 mL medium, of which 19 mL was hydrolyzate (or, for the references, glucose solution in water), sealed with rubber stoppers, and equipped with cannulas for carbon-dioxide removal. The flasks were inoculated to an initial cellmass concentration of 2 g/L DW and incubated at 30°C with stirring. The fermentations were run for 36 h under nonsterile conditions.

Samples of 100 µL were withdrawn before inoculation and after 2, 4, 6, 8, 12, 24, and 36 h. Seven detoxified samples were run in each fermentation experiment together with two reference fermentations. Each fermentation was repeated at least three times.

The fermentability was evaluated as ethanol yield calculated as produced ethanol divided by consumed amount of fermentable sugars (Y_{EtOH} [g/g]) and the maximum mean volumetric productivity, hereafter referred to as volumetric productivity, calculated as ethanol produced within the first 6 h of fermentation divided by 6 (Q_{6h} [g/L/h]), because the maximum mean volumetric productivity was obtained after 6 h in the reference fermentations.

Anaerobic growth yield, hereafter referred to as biomass yield, was calculated as produced biomass in 24 h (DW) divided by consumed amount of fermentable sugars (Y_X [g/g]).

Analysis of Fermentation Products

In all fermentation samples, glucose, ethanol, lactic acid, and glycerol were separated by HPLC (Shimadzu) with a refractive index detector (RID-6A, Shimadzu) using an Aminex HPX-87H column (Bio-Rad) at 45°C, with 5 m*M* H_2SO_4 as the mobile phase, at a flow rate of 0.6 mL min^{-1}. The system was equipped with a Cation-H Refill Cartridge (Bio-Rad) prior to the HPX-87H column. Samples were diluted and filtered through 0.2-µm membrane filters (Advantec MFS, Pleasanton, CA) prior to analysis.

DW was determined at the beginning and the end of the fermentation. For DW determinations, nitro-cellulose filters with 0.45-µm pore size (Gelman Sciences, Ann Arbor, MI) were used. The cells were washed with Millipore water and dried in a microwave oven (Zanussi ZM23) at a power scale of 3.5.

Results

The hydrolyzate contained 32.2 g/L fermentable sugars (glucose and mannose) (Table 1). Among the furan derivatives, 5-HMF was the most abundant (5.9 g/L). The concentrations of the aliphatic acids ranged from 1.6 to 2.6 g/L. Fourteen different phenolic compounds were identified (Nilvebrant, N.O., unpublished data) and quantified (Table 1).

Table 1
Compounds Determined in the Dilute Acid Hydrolyzate of Spruce[a]

Group of compounds	Compounds[b]	Concentration (g/L)
Sugars	Glucose	25.7
	Mannose	6.5
	Galactose	3.7
	Xylose	3.5
	Cellobiose	0.7
	Arabinose	0.6
Furan derivatives	5-hydroxy-methyl-furfural	5.9
	Furfural	1.0
Aliphatic acids	Levulinic acid	2.6
	Acetic acid	2.4
	Formic acid	1.6
Phenolic compounds	Vanillin	0.12
	Dihydroconiferylalcohol	0.098
	Coniferyl aldehyde	0.035
	Vanillic acid	0.034
	Hydroquinone	0.017
	Catechol	0.009
	Acetoguaiacone	0.007
	Homovanillic acid	0.005
	4-Hydroxy-benzoic acid	0.005
Hibbert's ketones	G*-$CHOHCOCH_3$	0.048
	G*-$COCOCH_3$	0.029
	G*-CH_2COCH_2OH	0.028
	G*-$COCHOHCH_3$	0.025
	G*-CH_2COCH_3	0.016

[a]Produced with residence time, 7 min; reaction temperature, 222°C; and 0.5% sulfuric acid (w/w).
[b]G*, guaiacyl = 4-hydroxy-3-methoxyphenyl.

Twelve different detoxification treatments were performed (Table 2, Nos. 3–13,15). The highest ethanol yields were achieved after the anion-exchange treatment at pH 10 (0.49 g/g), treatment with laccase (0.47 g/g), anion-exchange treatment at pH 5.5 (0.45 g/g), and treatment with $Ca(OH)_2$ (0.44 g/g). The productivity, however, was for all treatments lower than in the reference fermentation, which contained only glucose and nutrients (1.46 g/L/h). The highest productivity was observed after anion exchange at pH 10 (1.42 g/L/h) and treatment with $Ca(OH)_2$ (1.21 g/L/h). Ethanol productivity was more than twice as high after treatment with $Ca(OH)_2$ compared with treatment with NaOH. Biomass yield was not comparable to the reference fermentation after any of the detoxification treatments. The highest biomass yield (0.080 g/g) was obtained after anion exchange at pH 10.

Small decreases (3–6%) in concentrations of fermentable sugars (glucose and mannose) were observed for all treatments owing to dilution with

Table 2
The Influence of the Detoxification
on the Fermentability by Baker's Yeast *Saccharomyces cerevisiae*

No	Detoxification method	Y EtOH (g/g)	Q 6 h (g/L/h)	Yx (g/g)
1	Reference fermentation	0.45 ± 0.004	1.46 ± 0.021	0.090 ± 0.001
2	None[a]	0.32 ± 0.004	0.04 ± 0.015	0.010 ± 0.001
3	pH 10 (sodium hydroxide)	0.42 ± 0.005	0.46 ± 0.019	0.015 ± 0.001
4	pH 10 (calcium hydroxide)	0.44 ± 0.007	1.21 ± 0.065	[b]
5	pH 5.5, 0.1% sulfite	0.34 ± 0.006	0.09 ± 0.035	0.020 ± 0.001
6	pH 10, 0.1% sulfite	0.42 ± 0.004	0.45 ± 0.015	0.030 ± 0.001
7	pH 5.5, 1% sulfite	0.42 ± 0.005	0.45 ± 0.017	0.020 ± 0.001
8	pH 10, 1% sulfite	0.43 ± 0.004	0.47 ± 0.016	0.040 ± 0.002
9	Evaporation of 10%	0.34 ± 0.006	0.06 ± 0.017	0.015 ± 0.001
10	Evaporation of 90%	0.42 ± 0.010	0.33 ± 0.026	0.030 ± 0.002
11	pH 5.5, anion exchange	0.45 ± 0.008	0.66 ± 0.052	0.060 ± 0.010
12	pH 10, anion exchange	0.49 ± 0.010	1.42 ± 0.040	0.080 ± 0.005
13	Laccase	0.47 ± 0.004	0.68 ± 0.007	0.055 ± 0.003
14	Laccase control	0.32 ± 0.006	0.05 ± 0.032	0.012 ± 0.001
15	*T. reesei*	0.43 ± 0.005	0.55 ± 0.070	0.055 ± 0.005
16	*T. reesei* control	0.42 ± 0.006	0.17 ± 0.035	0.030 ± 0.003

[a]pH was adjusted to 5.5 with NaOH prior to the fermentation.
[b]Not determined owing to the precipitation.

either acid or base when pH was adjusted to 5.5 or 10 (Table 3). The concentration of fermentable sugars decreased negligibly when pH was adjusted to 10 with either NaOH or $Ca(OH)_2$ (4%) compared to when pH was adjusted to 5.5 (3%). The highest decrease in the concentrations of fermentable sugars was observed after treatment with *T. reesei* (35%), anion exchange at pH 10 (26%) and anion exchange at pH 5.5 (8%) (Table 3). The concentration of aliphatic acids was not largely affected by detoxification (Table 3). The concentration of levulinic acid decreased only after anion-exchange treatments. Levulinic acid was not as volatile as acetic and formic acid, which were partially removed also by evaporation of 90% of the initial volume. Most of the applied detoxification methods decreased the concentration of the furan derivatives furfural and 5-HMF. Furfural was partially removed by evaporation of 10% of the initial volume and completely removed by evaporation of 90% of the initial volume, whereas the concentration of 5-HMF decreased only by 4% in the latter case (Table 3). In addition, the concentration of furfural was greatly decreased by the fungal fermentation; however, the same trend was observed in the control treatment. About 70% of both furan derivatives was removed by anion exchange at pH 10 and 37–53% was removed by treatment with 1% sulfite at pH 5.5 or 10. Treatment with 0.1% sulfite at pH 5.5 did not affect the concentration of furan derivatives.

The total concentration of phenolic compounds was affected by all detoxification methods applied. Phenolics were most efficiently removed

Table 3
The Influence of Detoxification on the Composition of the Hydrolyzate[a]

Method	Fermentable sugars (glucose + mannose)	Levulinic acid	Acetic acid	Formic acid	Furfural	5-HMF	Total phenolic content (GC-MS)	Total phenolic content (Prussian blue)[b]
None	97	100	100	100	100	100	100	100
pH 10 (NaOH)	96	100	100	100	81	84	82	96
pH 10 ($Ca(OH)_2$)	96	100	100	100	80	78	81	81
pH 5.5, 0.1% sulfite	96	100	100	100	100	100	92	ND
pH 10, 0.1% sulfite	95	100	100	100	88	89	96	ND
pH 5.5, 1% sulfite	96	100	100	100	61	63	92	ND
pH 10, 1% sulfite	96	100	100	100	47	48	81	ND
Evaporation of 10%	97	100	100	100	63	100	92	99
Evaporation of 90%	97	100	35	26	0	96	98	85
pH 5.5, anion exchange	92	14	11	8	69	74	31	29
pH 10, anion exchange	74	7	4	3	27	30	9	9
Laccase	96	100	100	100	100	100	20	7
Laccase control	96	100	100	100	100	100	100	100
T. reesei	65	100	100	100	15	75	94	95
T. reesei control	94	100	100	100	24	81	100	100

[a]Table numbers are expressed as percentage of the concentration of compounds left after detoxification.
[b]ND, not determined, owing to the interference of sulfite with the assay.

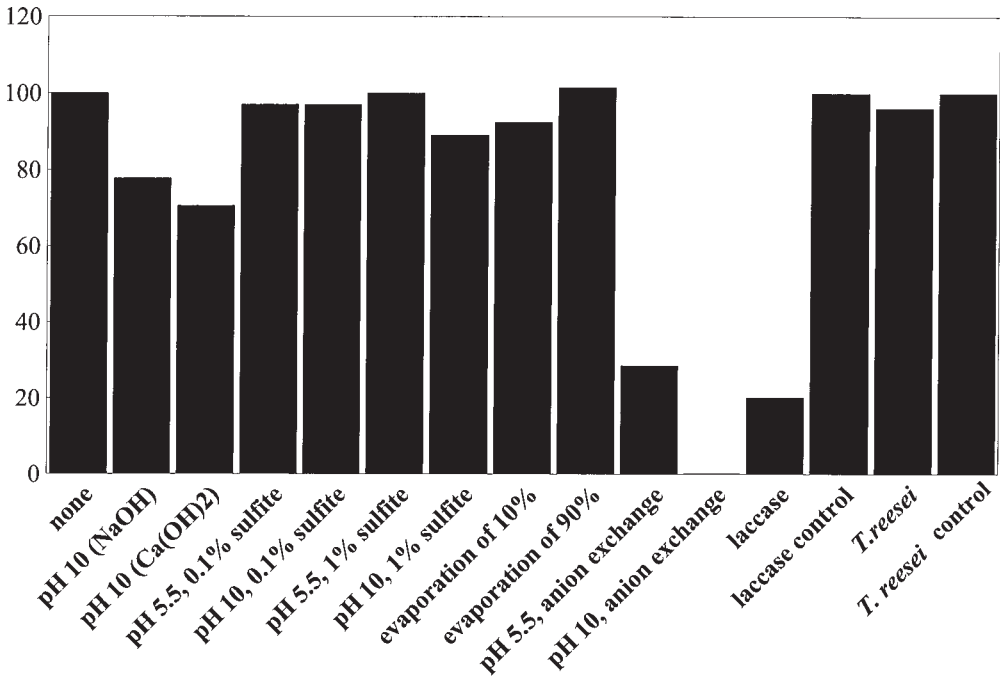

Fig. 1. The decrease in the concentration of Hibbert's ketones in the detoxified hydrolyzate.

by treating with laccase and anion exchange at pH 5.5 and 10. Total concentration of phenolic compounds in the hydrolyzate detoxified with sulfite could not be determined with the Prussian blue method because sulfite interfered with the assay. The total amount of phenolics in the untreated hydrolyzate was determined to 0.64 g/L by GC-MS analysis, whereas according to the Prussian blue assay it was 1.86 g/L.

The Hibbert's ketones were studied separately (Fig. 1; Table 1), because related compounds—according to the study of Clark and Mackie *(15)*—may be important inhibitors in softwood hydrolyzates. The three methods, which were efficient for decreasing the total amount of phenolic compounds (anion exchange at 5.5, anion exchange at pH 10, and treatment with laccase) were also the most efficient methods to remove the Hibbert's ketones.

Discussion

Improved fermentability was observed for all detoxification methods used in the present study; however, there were major differences between the efficiency of the detoxification methods as well as for the groups of inhibitory compounds removed by each method.

Detoxification of lignocellulose hydrolyzates by alkali treatment, i.e., increasing pH to 9–10 with NaOH or $Ca(OH)_2$ followed by pH readjustment to 5.5 with H_2SO_4, is a well-known method to increase fermentability

(20). Adjustment of pH with $Ca(OH)_2$ has been reported to increase the fermentability more than adjustment with NaOH *(19)*, which agrees with our results. There was, however, only little difference in the amounts of measured compounds (Table 3). According to the measurements with the Prussian blue method, the total amount of phenolic compounds was more efficiently decreased by $Ca(OH)_2$. However, it has been shown that monovalent ions such as Na^+ affect the ethanol productivity negatively, whereas Ca^{2+} does not *(19)*. This could explain the higher productivity observed in hydrolyzates detoxified with $Ca(OH)_2$ in this study. However, in contrast to what has been reported previously, neither acetic acid *(19,25,28)* nor sugars *(25)* were removed by treatment with NaOH or $Ca(OH)_2$.

Treatment with reducing agents, such as sulfite, has also been reported to be an efficient detoxification method *(20,23,29)*. The results show that to improve the fermentability a sulfite concentration higher than 0.1% is necessary. The addition of 0.1% sulfite is not sufficient to affect the amounts of furan derivatives if calculated on a molar basis, whereas the addition of 1% sulfite results in a slight excess.

It has previously been shown that including a heating step in the overliming procedure (leading to some evaporation) improves fermentability *(28)*. Our results show that fermentability was increased after evaporation, but that the improvement was rather limited. The results indicate that volatile compounds are not the major inhibitors, which agrees with previous investigations *(30)*.

Previously, the filamentous fungus *T. reesei* has been shown to improve the fermentability of a hemicellulose hydrolyzate of willow *(24)*. In this study, it was shown that the fungus is also capable of improving the fermentability of a dilute-acid hydrolyzate of spruce. In contrast to the previous report *(24)*, the treatment did not affect the concentration of acetic acid. This was probably due to slower fungal growth, as indicated by the sugar consumption, perhaps because of the different nature of the hydrolyzate used in the present study. The concentrations of furan derivatives decreased; however, mostly owing to evaporation, as shown by the control treatment. The pH in the *T. reesei* control was 6.0 and the aliphatic acids should be less volatile than in the evaporation treatment, which was performed prior to the adjustment of pH to 5.5. The pH can account for why the concentrations of aliphatic acids did not decrease in the *T. reesei* control, whereas the concentration of furan derivatives did.

Treatment with anion-exchange resin at pH 10 gave the best ethanol yield, productivity, and biomass yield owing to a significant decrease in the concentrations of all three groups of inhibitors. This method, however, also removed 26% of the fermentable sugars (glucose and mannose) in the hydrolyzate, which is a major drawback. The decrease in sugar concentration was most probably due to hydrophobic interaction with the polystyrenedivinylbenzene-based matrix. Treatment with laccase affected only the concentration of phenolic compounds and was the only method that was specific for one group of inhibitors. The ethanol yield was only slightly

lower than that obtained after anion exchange at pH 10; however, the ethanol productivity and the biomass yield were still lower than in the reference fermentation (47 and 61%, respectively).

Two different methods for measuring the total concentration of phenolics show different results in some cases (Table 3). This discrepancy may be due to that only a limited number of the phenolic compounds were determined by GC-MS, whilst the spectrophotometric method may also determine phenols with higher molecular mass. The data supports this; the total amount of phenolic compounds in the hydrolyzate was 0.64 g/L as determined by GC-MS, in comparison with 1.86 g/L as determined by using the Prussian blue method. The decrease in the amount of Hibbert's ketones at pH 10 was lower in the presence of sulfite (Fig. 1). This may be because in the presence of sulfite oxidation was prevented, and because the treatment was done under an atmosphere of helium.

A higher ethanol yield than in the reference fermentation was observed after laccase treatment. This could be owing to aliphatic acids, which were still present after detoxification. The presence of weak acids probably also contributed to the higher ethanol yield after anion-exchange treatment. This is supported by the biomass yield being lower than in the reference fermentation. Furan derivatives were not removed by laccase, which resulted in an initial lag phase in ethanol production. This is the most probable explanation why the productivity and biomass yield did not reach that of the reference fermentation. The anion exchange at pH 10 also removed most of the furan derivatives, probably owing to hydrophobic interactions with the anion-exchange matrix, which resulted in higher productivity and biomass yield.

The results indicate that good fermentability can be achieved if phenolic compounds are removed from the hydrolyzate. Low concentrations of weak acids can have a slightly beneficial effect on ethanol yield at the expense of biomass formation. A combination of laccase treatment and evaporation could be of interest because it would allow the removal of furfural, which would increase ethanol productivity. The use of $Ca(OH)_2$ instead of NaOH for pH adjustment to 5.5 prior to the detoxification treatment may also improve productivity.

However, the choice of detoxification method must be made after considering the composition of hydrolyzate and type of raw material. The composition of softwood and hardwood hydrolyzates differs, so an appropriate method must be chosen to remove relevant groups of inhibitors. For instance, it has been shown that hardwood hydrolyzates contain higher concentrations of acetic acid and furfural than softwood hydrolyzates *(26)*. A method that removes weak acids and furfural, such as evaporation of 90% initial volume, could be more effective for a hardwood hydrolyzate than a softwood hydrolyzate.

Knowledge of the chemical effects of different detoxification methods may be helpful for rational design of detoxification procedures to achieve good fermentability of a selected hydrolyzate.

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