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Effect of Different Forms of Alkali Treatment on Specific Fermentation Inhibitors and on the Fermentability of Lignocellulose Hydrolysates for Production of Fuel Ethanol

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Treatment with alkali, particularly overliming, has been widely used as a method for the detoxification of lignocellulose hydrolysates prior to ethanolic fermentation. However, the mechanisms behind the detoxification effect and the influence of the choice of cation have not been well understood. In this study, a dilute acid hydrolysate of spruce and an inhibitor cocktail consisting of six known inhibitors were used to investigate different alkali detoxification methods. The various treatments included the addition of calcium hydroxide, sodium hydroxide, potassium hydroxide, and ammonia to pH 10.0 and subsequent adjustment of the pH to 5.5 with either sulfuric or hydrochloric acid as well as treatment with the corresponding amounts of calcium, sodium, and potassium as sulfate or chloride salts at pH 5.5. An RP-HPLC method was developed for the separation of 18 different inhibitors in the hydrolysate, including furaldehydes and phenolics. Detection and quantification were carried out by means of UV, DAD, and ESI-MS in negative mode. Treatment of the spruce hydrolysate with alkali resulted in up to ~40% decrease in the concentration of furaldehydes. The effects on the aromatic compounds were complex. Furthermore, SFE was performed on the precipitate formed during alkali treatment to evaluate the inhibitor content of the precipitate, and the following RP-HPLC analysis implied that potential inhibitors were removed mainly through conversion rather than through filtration of precipitate. Parallel experiments in which sulfuric acid or hydrochloric acid was used for acidification to pH 5.5 after alkali treatment indicated that the choice of anion did not affect the removal of inhibitors. Detoxification with calcium hydroxide and ammonia resulted in better fermentability using *Saccharomyces cerevisiae* than detoxification with sodium hydroxide. The results from the experiments with the inhibitor cocktail indicated that the positive effects of alkali treatment are difficult to explain by removal of the inhibitors only and that possible stimulatory effects on the fermenting organism warrant further attention.

KEYWORDS: Detoxification; alkali; inhibitors; lignocellulose hydrolysates; fuel ethanol; *Saccharomyces cerevisiae*

INTRODUCTION

Ethanol produced from renewable substrates (*1*) has gained interest as an oxygenated gasoline additive. Environmental concerns have been raised regarding the use of methyl *tert*-butyl ether (MTBE) as an oxygenator in fuel (*2*). In addition, fuels produced from renewable resources do not contribute to a net increase in carbon dioxide in the atmosphere. Lignocellulosic biomass, for example, forestry wastes, is an abundant renewable resource that is needed to produce sufficient amounts of low-cost ethanol in addition to conventional raw materials, such as starch from agricultural crops and sugar cane juice (*1*).

To obtain fermentable monomeric sugars from lignocellulose, the hemicellulose and the cellulose need to be hydrolyzed. Acid hydrolysis has proven to be a fast and relatively cheap method for acquiring sugars from lignocellulose (*3*, *4*). However, in addition to sugars, byproducts including aliphatic acids, such as acetic, formic, and levulinic acid, furaldehydes, and phenolic compounds are formed. These byproducts originate from the degradation of cellulose, hemicellulose, and lignin. Some of the byproducts have been found to be toxic to the fermenting organism, and high concentrations result in decreased ethanol productivity and yield (*5*−*13*). The character and concentrations of these inhibitors may differ significantly depending on the pretreatment and hydrolysis conditions as well as on the raw

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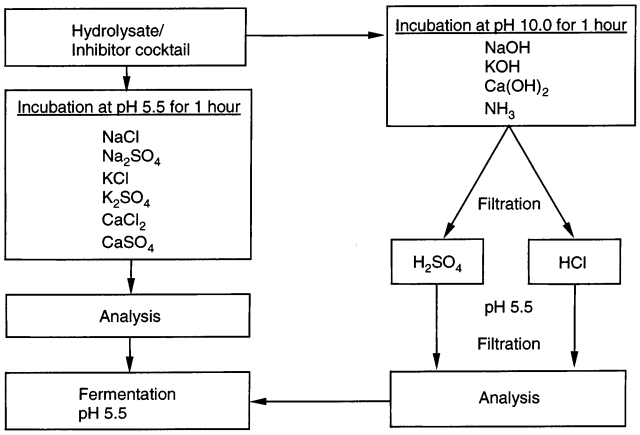

Figure 1. Schematic sequence of the treatments of the hydrolysate and the inhibitor cocktail, respectively.

material (*14*). To facilitate fuel ethanol production from lignocellulose hydrolysates, detoxification methods have therefore been developed (*9*−*11*, *13*, *15*). Furthermore, to evaluate and improve different hydrolysis as well as detoxification methods it is of great importance to be able to identify and quantify the inhibitors that are present in the hydrolysates.

Alkali treatment, particularly treatment with calcium hydroxide (overliming), is a widely used method to improve the fermentability of dilute acid lignocellulose hydrolysates (*9*, *10*, *13*, *15*, *16*). Up to now it has also been proven to be one of the best methods for detoxification (*15*). However, knowledge of the chemistry behind the detoxification process has been very limited. Very recently, a few studies taking the chemical effects of alkali treatment into consideration have been performed (*9*, *10*, *13*, *15*). Although alkali treatment is known to affect the concentration of toxic compounds, for instance, furaldehydes and phenolics, the explanations behind the great improvement in fermentability remain to be revealed. In addition, treatment with calcium hydroxide has been found to result in better improvement of fermentability than treatment with sodium hydroxide, but the difference in effect between various forms of alkali has not been understood.

In this work, the effects of different forms of alkali treatment were investigated with regard to the removal of specific inhibitors present in a two-step dilute acid hydrolysate prepared from Norway spruce and in a synthetic inhibitor cocktail containing six selected toxic compounds representing different types of inhibitors: two aliphatic acids (formic and acetic acid), two furaldehydes [2-furaldehyde and 5-(hydroxymethyl)-2-furaldehyde (HMF)], and two phenolics (coniferyl aldehyde and ferulic acid) (**Figure 1**). The inhibitor cocktail was included as a model lignocellulose hydrolysate, toxic to the fermenting microorganism but with lower complexity than an authentic hydrolysate, with the aim to have a system in which all inhibitory compounds were known and could be readily measured. Moreover, the effect of using either sulfuric acid or hydrochloric acid for acidification after treatment at alkaline conditions was compared. Reversed phase high-performance liquid chromatography (RP-HPLC) coupled to ultraviolet (UV) detection, diode array detection (DAD), and electrospray ionization mass spectrometry (ESI-MS) enabled direct qualitative and quantitative determination of furaldehydes and phenolic compounds without any time-consuming sample pretreatment. Hydrolysate and inhibitor cocktail samples, treated with different alkali and salts, were fermented using ordinary baker's yeast, *Saccharomyces cerevisiae*. The effects of the different treatments on the chemical composition as well as on the fermentability were thus compared (**Figure 1**). This experimental approach was designed to account for effects on the concentration of inhibiting compounds as well as potential positive and negative effects on the fermenting microorganism.

Supercritical fluid extraction (SFE) was used to extract inhibitors from the precipitate after selected detoxification procedures. The SFE experiments were performed to elucidate whether the decreased concentration of inhibitors after detoxification was due to adsorption to the precipitate or degradation of the individual compounds.

MATERIALS AND METHODS

Chemicals. Carbon dioxide for extraction (purity > 99.998%) was delivered by AGA Gas AB (Lidingö, Sweden). Methanol (pro analysi, p.a.), formic acid (p.a.), acetonitrile (HPLC quality), 4-hydroxybenzoic acid (for synthesis), phenol (p.a.), vanillin (4-hydroxy-3-methoxybenzaldehyde) (for synthesis), guaiacol, calcium hydroxide (p.a.), sodium chloride (p.a.), potassium chloride (p.a.), calcium chloride (p.a.), calcium sulfate (p.a.), ammonia (25%, p.a.), sulfuric acid (95−97%, p.a.), and hydrochloric acid (37%, p.a.) were supplied by Merck (Darmstadt, Germany). Coniferyl aldehyde (98%), ferulic acid, α-hydroxyguaiacone, 3,4-dihydroxybenzoic acid (97%), 3,4-dihydroxybenzaldehyde (97%), 5-(hydroxymethyl)-2-furaldehyde (HMF) (99%), and syringic acid were purchased from Sigma-Aldrich (Steinheim, Germany). Coniferyl alcohol (99%), *trans*-cinnamaldehyde (p.a.), and 4-hydroxybenzaldehyde were supplied by Acros Organics (Geel, Belgium). Catechol, 2-furaldehyde, and vanillic acid were from ICN Biomedicals Inc. (Aurora, OH). Potassium sulfate (p.a.) and cinnamic acid (≥99%) were from Fluka (Buchs, Switzerland). Sodium sulfate was delivered by Riedel-de-Haën (Seelze, Germany). Sodium hydroxide and potassium hydroxide (pellets, p.a.) were from Eka Nobel (Bohus, Sweden). All water was of Milli-Q quality (Millipore, Bedford, MA).

Media. The detoxification experiments were performed on both the lignocellulose hydrolysate and the inhibitor cocktail according to the scheme in **Figure 1**. A two-step dilute acid hydrolysate of Norway spruce (*Picea abies*) was used. Chipped Norway spruce was impregnated with sulfuric acid (0.5% w/v) prior to the loading in a 250-L batch reactor. Steam at a pressure of 12 bar (190 °C) was loaded and kept for 10 min. Subsequently, the liquid and solid fractions were separated, after which the solid fraction was washed with water, reimpregnated with sulfuric acid, and loaded into the reactor again. Steam at a pressure of 21 bar (215 °C) was loaded and kept for 10 min. After filtration, the liquid fractions from steps 1 and 2 were pooled to form the final hydrolysate. The pH value of the final hydrolysate was 1.9.

The inhibitor cocktail contained 75 mM (3.45 g/L) formic acid, 75 mM (4.50 g/L) acetic acid, 30 mM (2.88 g/L) 2-furaldehyde, 30 mM (3.78 g/L) HMF, 5.0 mM (0.97 g/L) ferulic acid, 1.0 mM (0.18 g/L) coniferyl aldehyde, and 188 mM (35.0 g/L) glucose. The pH during alkali and salt treatment was monitored using a pH-meter from Radiometer (PHM 83 Autocal pH meter, Radiometer, Copenhagen, Denmark).

Alkali Treatments. Fifty milliliters of the hydrolysate (pH 1.9) or, alternatively, the inhibitor cocktail (pH 2.2) was adjusted to pH 10.0 with 5.0 M NaOH, 5.0 M KOH, 2.5 M $Ca(OH)_2$, or 25% NH_3, respectively. The samples were stirred for 1 h at room temperature and thereafter vacuum filtered using 0.45 μm membrane filters of nitrocellulose (type HA) (Millipore). Concentrated acid (sulfuric acid or hydrochloric acid) was added until the pH reached 5.5, as all fermentations were performed at this pH value. Then, the samples were filtered once more. See **Table 1** (hydrolysates 1−8 and inhibitor cocktails 15−22) for an overview.

Salt Treatments. Initially, the pH of the hydrolysate (1.9) and inhibitor cocktail (2.2) was adjusted to 5.5 with the hydroxide of the cation of the salt, for example, sodium hydroxide was used to adjust the pH to 5.5 before sodium chloride was added, etc. After the pH adjustment, the remaining amount of cations was added as 5.0 M NaCl, 2.5 M $CaCl_2$, or 2.5 M $CaSO_4$, respectively. K_2SO_4, Na_2SO_4, or KCl was added in solid form. The different salts were added so that the final concentration of cations in the samples corresponded to the total

Table 1. Overview of the Different Treatments of the Hydrolysate and the Inhibitor Cocktail[a]

sample					
hydrolysate	inhibitor cocktail	base	pH	acid to pH 5.5	salt
1		NaOH	10.0	H_2SO_4	
2		NaOH	10.0	HCl	
3		$Ca(OH)_2$	10.0	H_2SO_4	
4		$Ca(OH)_2$	10.0	HCl	
5		KOH	10.0	H_2SO_4	
6		KOH	10.0	HCl	
7		NH_3	10.0	H_2SO_4	
8		NH_3	10.0	HCl	
9		NaOH	5.5		Na_2SO_4
10		NaOH	5.5		NaCl
11		$Ca(OH)_2$	5.5		$CaSO_4$
12		$Ca(OH)_2$	5.5		$CaCl_2$
13		KOH	5.5		K_2SO_4
14		KOH	5.5		KCl
	15	NaOH	10.0	H_2SO_4	
	16	NaOH	10.0	HCl	
	17	$Ca(OH)_2$	10.0	H_2SO_4	
	18	$Ca(OH)_2$	10.0	HCl	
	19	KOH	10.0	H_2SO_4	
	20	KOH	10.0	HCl	
	21	NH_3	10.0	H_2SO_4	
	22	NH_3	10.0	HCl	
	23	NaOH	5.5		Na_2SO_4
	24	NaOH	5.5		NaCl
	25	$Ca(OH)_2$	5.5		$CaSO_4$
	26	$Ca(OH)_2$	5.5		$CaCl_2$
	27	KOH	5.5		K_2SO_4
	28	KOH	5.5		KCl

[a] All samples that were treated at pH 10.0 were adjusted to pH 5.5 with either sulfuric or hydrochloric acid prior to fermentation. See **Figure 1** for an overview of the experimental procedure.

amount of cations added in the alkali treatments at pH 10.0. The samples were stirred for 1 h at room temperature, after which time the samples were vacuum filtered. See **Table 1** (hydrolysates 9−14 and inhibitor cocktails 23−28) for an overview.

SFE of Membrane Filters. SFE of the solid material captured on the membrane filters from the first filtration step (**Figure 1**) was performed for three selected filters. The three filters were selected for extraction after an ocular inspection. The selected filters contained solid material obtained after treatment of the hydrolysate at pH 10.0 with NaOH, $Ca(OH)_2$, or NH_3. An ISCO SFX 3560 automated supercritical fluid system with a model 260D syringe pump (Lincoln, NE) was used for the extractions. All extractions were performed in dynamic mode at 270 bar and 40 °C for 60 min. Furthermore, the analytes were collected in pressurized methanol at 10 °C with an addition of 0.5 mL of methanol every 5 min. The CO_2 flow was 2.0 mL/min, and the restrictor temperature was set to 50 °C.

HPLC Analysis. The analysis of the furans and the phenolic compounds (**Table 2**) was performed using an HP 1100 series HPLC system equipped with a binary pump, an autoinjector, and a variable-wavelength detector (VWD; Hewlett-Packard, Palo Alto, CA). Furthermore, the HPLC system was connected to an HP 1050 series DAD and an Esquire-LC ion-trap mass spectrometer with an atmospheric pressure ionization (API)−electrospray interface operated in negative ionization mode (Bruker Daltonics, Bremen, Germany). An XTerra MS C_{18}, 5 μm, 2.1 × 150 mm analytical column with an XTerra MS C_{18}, 5 μm, 2.1 × 10 mm guard column (Waters, Milford, MA) was used for the separation. The eluent was a gradient of Milli-Q water (Millipore) and acetonitrile, both of which contained 2 mM formic acid. The mobile phase gradient started with 5% acetonitrile for 5 min, after which time the acetonitrile content increased linearly to 10% after 10 min, to 30% after 20 min, to 50% after 40 min, and finally it was kept at 50% acetonitrile until 60 min.

The mass spectrometer was set to scan between *m*/*z* 50 and 210. Nitrogen was used as the drying gas and was pumped into the interface at a rate of 7 L/min and at a temperature of 350 °C. Nitrogen was also used as the nebulizer gas and was kept at 30 psi. The following voltages were used: nebulizer capillary tip, 4900 V; endplate at the sampling orifice, 4200 V; sampling capillary exit, −60 V; $skimmer_1$, −30 V; and $skimmer_2$, −8 V.

External calibration curves (five points) for all individual analytes were used for the quantification. Standard addition and wavelength spectra were sometimes necessary to use for the qualitative determination. The wavelength for quantification was selected on the basis of the absorption maximum of the compound and the interference from coeluting compounds (**Table 2**). Mass spectrometric detection was selected for quantification of 4-hydroxybenzaldehyde as there were many coeluting compounds at that retention time (vanillic acid and syringic acid, **Table 2**; **Figure 2**), and 4-hydroxybenzaldehyde gave a good signal in the mass spectrometer. Furthermore, the hydrolysates had to be diluted to concentrations suitable for the analytical system when HMF, 2-furaldehyde, and vanillin were analyzed.

Analysis of Calcium Content. The concentration of calcium was determined, after acidification with nitric acid to 2% (v/v), with inductively coupled plasma spectrometry (ICP-OES) using an Optima 2000 DV from Perkin-Elmer (Boston, MA).

Analysis of Aliphatic Acids. The aliphatic acids in the inhibitor cocktail (formic and acetic acid) were measured with a high-performance anion exchange chromatography (HPAEC) system consisting of a Dionex DX-500 series ion chromatograph equipped with an ED-40 conductivity detector and an ASRS-1 membrane suppressor (Dionex, Sunnyvale, CA). The acids were separated on an IonPac AS11-HC (250 mm × 4 mm i.d.) analytical column equipped with an AG11-HC guard column (Dionex) using isocratic elution. The eluent consisted of 0.91 mM NaOH and 7.5% (v/v) methanol, and the flow rate was 1.4 mL/min. A solution of 100 mM NaOH was used to wash the column between consecutive runs.

The aliphatic acids in the hydrolysate were instead measured using capillary electrophoresis (CE) to avoid problems with compounds coeluting with levulinic acid in the HPAEC analysis. A Beckman P/ACE MDQ system was used with a 50-cm capillary, i.d. 75 μm, at 25 °C and 20 kV reversed polarity. Detection was performed by indirect UV at 280 nm. A trimellitic acid buffer was prepared from 0.26 g of trimellitic acid, 1.5 g of tris(hydroxymethyl)aminomethane, and 0.09 g of tetradecyl trimethylammonium bromide in 250 mL of Milli-Q water (Millipore). Before use, 47 mL of the buffer was mixed with 3 mL of 8.4 mM $CaCl_2$, filtered through a 0.2 μm cellulose nitrate filter, and degassed using He. Prior to each run, the capillary was conditioned with 0.1 M NaOH for 2 min, with Milli-Q water for 3 min, and with the buffer for 5 min. All operations were performed at 20 psi. The samples were diluted, neutralized if necessary, and filtered through a Whatman cellulose acetate filter with a pore size of 0.45 μm.

Fermentations. Selected hydrolysates and inhibitor cocktails were adjusted to pH 5.5 prior to fermentation (as described above under Alkali Treatments and Salt Treatments). The fermentability of the hydrolysates and the inhibitor cocktails was evaluated using 25-mL fermentors with an operating volume of 20 mL, of which a portion of 19 mL was the hydrolysate or the inhibitor cocktail solution. An inoculum of 4 g/L *S. cerevisiae* (baker's yeast, Jästbolaget AB, Rotebro, Sweden) was used in all fermentations. The hydrolysate and the inhibitor cocktail were supplemented with nutrients to the following final concentrations: 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 fermentors were sealed with rubber plugs and equipped with cannulae for carbon dioxide removal. The fermentations were run for 24 h at 30 °C with stirring (250 rpm) under oxygen-limited and non-aseptic conditions. All fermentations were performed in duplicates. Control fermentations with 35 g/L glucose, and additional nutrients as described above were performed in triplicates (on every fermentation occasion). Samples (500 μL) for analysis of consumed sugar and produced ethanol were taken at the beginning of the fermentations and then after 7 and 24 h.

Analysis of Fermentations. The sugars (fermentable hexoses, glucose and mannose; and a nonfermentable pentose, xylose) were determined by HPAEC with a Dionex DX-500 chromatography system

Table 2. Retention Times in the HPLC Separations, Molar Masses, and Selected Wavelengths for Quantification as well as the Concentrations of the Individual Compounds prior to Detoxification

	compound	t_R (min)	M (g/mol)	wavelength (nm)	concn in hydrolysate[a]	concn in inhibitor cocktail[b]
a	HMF	7.4	126.11	254	2.4	3.8
b	2-furaldehyde	9.7	96.09	254	0.7	2.9
c	3,4-dihydroxybenzoic acid	11.4	154.12	210	3.9	
d	catechol	14.3	110.11	280	3.5	
e	3,4-dihydroxybenzaldehyde	17.2	138.12	280	3.1	
f	4-hydroxybenzoic acid	18.4	138.12	210	41.0	
g	vanillic acid	20.5	168.15	254	15.7	
h	4-hydroxybenzaldehyde	21.0	122.12	(MS)	1.9	
i	syringic acid	21.3	198.17	254	16.6	
j	α-hydroxyguaiacone	21.9	196.20	254	11.8	
k	phenol	22.9	94.11	210	3.0	
l	vanillin	23.1	152.15	330	96.1	
m	coniferyl alcohol	23.1	180.20	254	9.0	
n	ferulic acid	24.8	194.20	254	1.7	1.0
o	guaiacol	25.1	124.14	254	3.3	
p	coniferyl aldehyde	26.8	178.20	280	41.8	0.2
q	cinnamic acid	31.6	148.16	254	1.1	
r	*trans*-cinnamaldehyde	33.2	132.16	254	0.2	

[a] Concentrations in g/L for compounds **a** and **b** and in ppm for all others. [b] Concentrations in g/L.

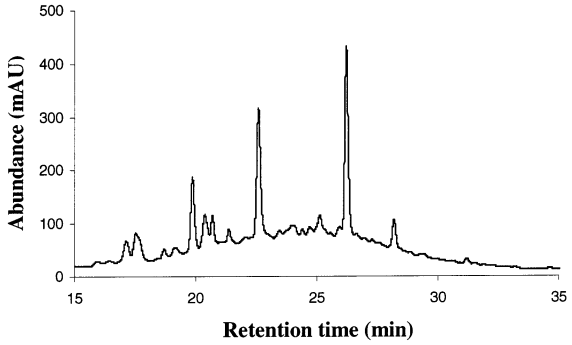

Figure 2. Part of UV chromatogram (254 nm) from an HPLC separation of a hydrolysate.

coupled with pulsed amperometric detection (PAD) (Dionex ED-40) and using a CarboPac PA-1 column (all from Dionex). The column was first equilibrated with a mixture of 200 mM NaOH and 170 mM NaAc for ∼5 min. After sample injection, an isocratic elution with pure water at a flow rate of 1.0 mL/min and postcolumn addition of 300 mM NaOH was applied. L-Fucose was used as an internal standard. The concentrations of glucose, mannose, and xylose were calculated using the EZchrom software system version 2.31 (Scientific Software Inc., Pleasanton, CA).

The concentration of ethanol was determined using an HP 5890 gas chromatograph (Hewlett-Packard) connected with an HP 7673A autoinjector (Hewlett-Packard) and equipped with a BP-20 column with a film thickness of 1.0 μm (SGE, Austin, TX) and an FID. The temperature was maintained at 35 °C for 5 min, which was followed by heating from 35 to 220 °C at a rate of 10 °C/min. Thereafter, the temperature was maintained at 220 °C for 10 min. Acetonitrile was used as the internal standard. The concentration of ethanol was calculated using the EZchrom software.

The dry weight of the yeast inoculum was determined using an Electronic Moisture Analyzer 40 (Sartorius AG, Göttingen, Germany). The moisture content of 1 mL of inoculum was determined using a temperature of 105 °C for 45 min.

RESULTS

HPLC Analyses of the Hydrolysate. All dilution steps during the treatments have been included in the calculations of the final concentrations of the furans and phenolics. The results from the HPLC analyses (**Figure 2**) of the specific furans and phenolic compounds in the hydrolysate have been summarized in **Table 3**. The values displayed in this table are relative concentrations after detoxification; the absolute concentrations before detoxification can be found in **Table 2**. Some general trends could be observed after the different detoxification methods; HMF, 2-furaldehyde, 3,4-dihydroxybenzaldehyde, 4-hydroxybenzaldehyde, and cinnamic acid decreased in concentration after all detoxifications, although it was more pronounced in the treatments at pH 10.0. The decrease in the concentration of HMF was ∼20% for sodium and potassium hydroxide but ∼30−40% for calcium hydroxide and ammonia. Although the difference was smaller, calcium hydroxide and ammonia treatments also gave lower concentrations of furfural than sodium and potassium hydroxide at pH 10.0. The concentration of 3,4-dihydroxybenzaldehyde decreased by 40−60% after treatment with alkali at pH 10.0, calcium hydroxide and ammonia being harsher. Moreover, the concentration of 4-hydroxybenzaldehyde decreased by 30−40% after treatment at pH 10.0 except for after treatment with ammonia, which resulted in a decrease of only ∼10%. The cinnamic acid concentration decreased by ∼40% after treatment with sodium and potassium hydroxide at pH 10.0, whereas a decrease of 80% could be observed after the calcium hydroxide and ammonia treatments. Furthermore, 4-hydroxybenzoic acid decreased slightly (10−20%) after all alkali treatments, and the concentration of guaiacol decreased after treatment with sodium (40−50%) and calcium hydroxide (30%) but not after treatment with potassium hydroxide and ammonia at pH 10.0 (**Table 3**). It should be noted that the concentration of phenol increased drastically after treatment with sodium, calcium, and potassium hydroxide, remained almost unaffected after all treatments at pH 5.5, but decreased considerably (20−40%) after treatment with ammonia at pH 10.0. The concentration of vanillin was not much affected by any of the treatments with the exception of ammonia at pH 10.0, after which roughly 10% remained. Due to the low concentration of *trans*-cinnamaldehyde (<0.2 ppm) the values in **Table 3** could not be established more accurately for this compound. However, it is clear that the concentration decreases by >50% after treatment with sodium hydroxide or potassium hydroxide and by ∼50% after treatment with calcium hydroxide at pH 5.5 and 10.0.

The treatments at pH 10.0 produced greater effects on the compounds in the hydrolysate than the treatments at pH 5.5.

Table 3. Relative Concentrations ($n = 2$) in the Hydrolysates, Compared to Absolute Values Stated in **Table 2**, as well as *F* Values from the Statistical Evaluation (ANOVA, $P = 0.05$) of the General Effect Treatment with either Base or Salt Had on the Concentrations of All Individual Compounds after the Different Detoxification Methods[a]

	compound																	
hydrolysate/ F_{crit}	HMF	2-furaldehyde	3,4-dihydroxybenzoic acid	catechol	3,4-dihydroxybenzaldehyde	4-hydroxybenzoic acid	vanillic acid	4-hydroxybenzaldehyde	syringic acid	α-hydroxyguaiacone	phenol	vanillin	coniferyl alcohol	ferulic acid	guaiacol	coniferyl aldehyde	cinnamic acid	*trans*-cinnamaldehyde[b]
untreated	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1	82	73	96	76	61	88	104	68	98	94	186	96	97	169	55	98	63	<50
2	81	73	84	91	66	87	108	67	97	103	175	96	84	154	63	99	60	<50
3	69	68	194	101	40	83	107	59	112	115	225	97	86	111	72	101	22	50
4	71	69	168	112	47	84	109	61	111	114	207	97	82	115	73	102	32	50
5	79	72	111	88	66	88	105	68	96	107	171	95	93	100	91	98	64	<50
6	81	74	94	85	64	89	106	71	94	105	164	96	85	102	97	98	69	<50
7	62	62	129	89	62	87	109	91	104	111	58	12	104	107	101	59	15	100
8	56	55	122	99	55	83	111	97	102	107	77	11	97	110	107	46	19	100
F_{alkali}	3248	769	23.8	1.9	82.5	85.2	4.8	31.9	19.3	12.5	213	1024	229	9.0	23.0	2434	2509	33.6
9	93	91	84	83	102	97	108	79	102	107	102	100	98	91	95	99	90	100
10	94	91	95	96	102	97	107	79	102	108	101	99	95	87	97	100	90	100
11	85	77	134	87	91	95	109	84	96	106	86	98	98	97	86	98	87	50
12	93	89	108	108	90	97	109	79	99	107	95	98	100	89	89	99	87	50
13	90	73	87	95	100	95	107	84	97	105	89	98	95	102	89	97	88	100
14	92	85	95	85	105	98	108	81	100	104	95	98	96	104	89	97	87	100
F_{salt}	262	154	3.3	1.5	3.4	13.8	16.0	10.8	4.1	3.4	3.4	73.6	18.9	7.0	19.5	32.9	62.3	8.3

[a] Treatments were as indicated in **Table 1**. *F* values above $F_{crit} = 3.23$ and $F_{crit} = 3.87$ for treatment with base or salt, respectively, imply that the detoxification methods were significant for the concentration of that individual compound. [b] Due to the low concentration of *trans*-cinnamaldehyde (<0.2 ppm) these values could not be established more accurately.

These effects include decreases (such as for HMF, 2-furaldehyde, 3,4-dihydroxybenzaldehyde, 4-hydroxybenzaldehyde, and cinnamic acid) as well as increases (phenol and ferulic acid) in concentration. Treatment with ammonia gave in many cases different results compared with treatment with the other forms of alkali at pH 10.0. These differences included decreases in the concentration of furaldehydes, phenol, vanillin, coniferyl aldehyde, and cinnamic acid.

Table 3 furthermore illustrates a statistical comparison (one-way ANOVA) for the treatments, either alkali at pH 10.0 or salts at pH 5.5. Samples detoxified with either alkali or salt were compared individually. When the obtained *F* values exceeded the critical *F* value, F_{crit}, for the individual compounds, then the choice of treatment was considered to be significant for the concentration of that compound. The higher the *F* value, the more important the selection of alkali or salt, respectively, will be. Only in the case of catechol did the alkali treatments of the hydrolysate not have any significant effect (95% confidence level) on the concentration of the inhibitor (**Table 3**). In contrast, for the salt treatments at pH 5.5 no significant effect on the concentrations was observed for many compounds in the hydrolysate, namely, 3,4-dihydroxybenzoic acid, catechol, 3,4-dihydroxybenzaldehyde, α-hydroxyguaiacone, and phenol (**Table 3**). The concentrations of all analyzed compounds were well above the detection limits except for *trans*-cinnamaldehyde (**Table 3**).

Analysis of Aliphatic Acids in the Hydrolysate. The concentrations of three aliphatic acids, formic, acetic, and levulinic acid, were determined in a selected number of samples using CE (**Table 4**). However, no major decrease in the concentration of any of the acids was detected.

Analysis of SFE Extracts from Precipitated Material. HMF, 2-furaldehyde, 4-hydroxybenzoic acid, vanillic acid, syringic acid, α-hydroxyguaiacone, phenol, vanillin, coniferyl alcohol, and coniferyl aldehyde were detected by HPLC analysis of the SFE extracts from the precipitated material collected by filtration of the alkali-treated hydrolysate prior to acidification (**Figure 1**). However, in all cases, the calculated amount captured on the filters corresponded to <1% of the amount that was originally present in the hydrolysate.

Table 4. Absolute (Grams per Liter, Where Indicated) and Relative Concentrations (Percent, All Others) of Aliphatic Acids in the Hydrolysate and Inhibitor Cocktail after Different Treatments (cf. **Table 1**)[a]

sample	formic acid	acetic acid	levulinic acid
hydrolysate	0.66 g/L	2.24 g/L	1.03 g/L
untreated[b]	100	100	100
1	110	102	100
3	109	100	110
7	103	112	103
9	107	108	104
11	101	106	99
inhibitor cocktail	3.45 g/L	4.50 g/L	
untreated[b]	100	100	
15	103	102	
17	97	96	
21	105	101	
23	103	100	
25	102	100	

[a] CE was used for analysis of the hydrolysate samples, whereas HPAEC was used for analysis of the cocktail samples. [b] pH was adjusted to 5.5 with NaOH.

HPLC Analyses of the Inhibitor Cocktail. The concentrations of specific furaldehydes and phenolic compounds in the inhibitor cocktail were determined by HPLC analyses, and the results are summarized in **Table 5**. The values displayed in this table are relative concentrations after detoxification. The absolute concentrations before detoxification can be found in **Table 2**. The concentrations of 2-furaldehyde and coniferyl aldehyde in the inhibitor cocktail decreased similarly by ~10% after all treatments. Furthermore, the alkali and salt treatments were significant (95% confidence level) for the concentration of all the compounds listed in **Table 5** according to the one-way ANOVA (**Table 5**).

Table 5. Relative Concentrations (Percent, $n = 2$) in the Inhibitor Cocktails, Compared to Absolute Values Stated in **Table 2**, as well as F Values from the Statistical Evaluation (ANOVA, $P = 0.05$) of the General Effect Treatment with either Base or Salt Had on the Concentrations of All Individual Compounds after the Different Detoxification Methods[a]

	compound			
inhibitor cocktail/F_{crit}	HMF	2-furaldehyde	ferulic acid	coniferyl aldehyde
untreated	100	100	100	100
15	104	90	107	94
16	99	82	101	94
17	103	88	102	94
18	101	85	98	83
19	104	88	108	89
20	103	86	100	94
21	98	84	98	94
22	101	85	83	89
F_{alkali}	61.9	321	5.4	15.0
23	98	87	95	89
24	104	92	99	89
25	98	88	96	89
26	99	89	96	94
27	97	88	97	89
28	97	87	98	89
F_{salt}	59.3	242	16.1	9.5

[a] F values above $F_{crit} = 3.23$ and $F_{crit} = 3.87$ for treatment with base or salt, respectively, imply that the detoxification methods were significant for the concentration of that individual compound.

The inhibitor cocktail contained acetic acid, formic acid, HMF, 2-furaldehyde, ferulic acid, and coniferyl aldehyde. These compounds were included in the cocktail at the grams per liter level. However, 3,4-dihydroxybenzaldehyde (2.1 ppm), 4-hydroxybenzoic acid (0.5 ppm), and cinnamic acid (0.4 ppm) were also detected in the inhibitor cocktail but in very low concentrations compared to the added compounds. These three compounds have possibly been formed in small amounts through degradation during the mixing and storage of the inhibitor cocktail or represent impurities in the chemicals.

Treatment of the hydrolysate at pH 10.0 resulted in more complex effects than treatment of the inhibitor cocktail. Treatment of the cocktail gave no clear differences between sodium hydroxide, calcium hydroxide, potassium hydroxide, and ammonia. Increase in the concentration of certain aromatic compounds, such as ferulic acid, was not observed in the cocktail. The concentration of HMF was not much affected in the cocktail, whereas there was a very clear decrease in the hydrolysate.

As in the case of the hydrolysate (**Table 3**), the choice of sulfuric acid or hydrochloric acid to adjust the pH to 5.5 after treatment at pH 10.0 did not show any clear trend with regard to the inhibitor concentrations (**Table 5**).

Analysis of Aliphatic Acids in the Inhibitor Cocktail. The concentrations of formic and acetic acid were determined in selected samples using HPAEC (**Table 4**). No removal of aliphatic acids by any of the treatments could be detected (**Table 4**). No ANOVA was performed on the results for aliphatic acids because so few samples were analyzed.

Analysis of Calcium Content. Analysis of the calcium content of selected samples (samples 3, 4, 11, 17, 18, and 25 and untreated hydrolysate, **Table 1**) showed that practically the same amount of calcium precipitates after pH adjustment to 5.5 with either sulfuric or hydrochloric acid. The concentrations of calcium in the hydrolysate were 2.70 and 2.44 g/L after treatment with $Ca(OH)_2/H_2SO_4$ or $Ca(OH)_2$/HCl, respectively, whereas the concentrations in the inhibitor cocktail were 3.09 and 2.70 g/L after treatment with $Ca(OH)_2/H_2SO_4$ or $Ca(OH)_2$/HCl, respectively. Furthermore, the concentrations of calcium in the hydrolysate and cocktail were 2.20 and 2.52 g/L, respectively, after treatment with $Ca(OH)_2/CaSO_4$ at pH 5.5. Hence, there was a slightly lower concentration of calcium in the samples after treatment at pH 5.5 than at pH 10.0. All measured values correspond well with the total amounts of added calcium, which were 3.34 g/L in the hydrolysate and 2.99 g/L in the cocktail. The original concentration of calcium in the hydrolysate was 0.12 g/L, whereas no calcium was added to the cocktail initially. Thus, ~10−20% of the calcium precipitates during the treatment and is filtered away.

Table 6. Fermentability and Sugar Content of the Hydrolysate (Glucose and Mannose) as well as the Inhibitor Cocktail (Glucose)[a]

sample	Glu + Man (%)	Y_{cons} (g/g)	Y_{tot} (g/g)	Q_{7h} (g/L/h)
reference[b]		0.40	0.40	1.38
hydrolysate				
untreated[c]	100	0.35	0.25	0.37
1	92	0.44	0.44	1.02
3	96	0.46	0.46	1.92
7	92	0.46	0.46	1.74
9	94	0.44	0.42	0.39
11	101	0.37	0.36	0.33
inhibitor cocktail				
untreated[c]	100	0.41	0.41	0.60
15	97	0.48	0.48	1.03
17	98	0.46	0.46	1.32
21	97	0.40	0.40	1.37
23	96	0.42	0.35	0.62
25	98	0.48	0.44	0.75

[a] Sugar concentrations were determined with an HPAEC system. Sugar content was ~35 g/L for the reference solution, hydrolysate, and inhibitor cocktail, respectively. Y_{cons} represents grams of formed ethanol per gram of consumed sugar, and Y_{tot} represents grams of formed ethanol per gram of totally available glucose and mannose. Q_{7h} represents grams of ethanol produced per liter of culture medium per hour during the first 7 h of fermentation. All numbers are mean values from two separate fermentations. [b] Nutrient solution in water. [c] pH was adjusted to 5.5 with NaOH.

HPAEC Analyses of Sugars. The results of the HPAEC analyses of sugars are displayed in **Table 6**. Not all samples (**Table 1**) were fermented as the HPLC analysis of the inhibitors indicated that the choice of using either hydrochloric acid or sulfuric acid for acidification was not critical (**Tables 3** and **5**). One disadvantage with the alkali treatment turned out to be a slight decrease in the total amount of fermentable sugars (**Table 6**). Initially, the sugar content was ~35 g/L for the reference solution, hydrolysate, and inhibitor cocktail, respectively. After the different treatments, at pH 5.5 and 10.0, the sugar content decreased by 4−8% in the hydrolysate and by 2−4% in the cocktail.

Fermentation Results. The results of the fermentations are displayed in **Table 6**. Under the conditions used, the productivities rather than the ethanol yields revealed major differences in fermentability. The most obvious difference was that samples treated at pH 10.0 showed higher fermentability than the samples treated at pH 5.5. This was true both for the hydrolysate and for the inhibitor cocktail. There was a 5-fold increase in productivity after treatment of the hydrolysate at pH 10.0 with calcium hydroxide or with ammonia (**Table 6**). These two samples performed better than even the reference fermentation. Treatment of the hydrolysate with sodium hydroxide at pH 10.0 resulted in a 3-fold increase in productivity. The samples treated

with calcium hydroxide and ammonia at pH 10.0 performed best also with regard to the inhibitor cocktail and showed a >2-fold increase in productivity (**Table 6**). Also in this case, the treatment with sodium hydroxide at pH 10.0 resulted in the third best productivity, after ammonia and calcium hydroxide.

DISCUSSION

Chemical Effects of Alkali and Salt Treatment of the Hydrolysate. The concentration of specific inhibitors in the hydrolysate varied considerably after the different treatments (**Table 3**). The concentrations of some of the compounds even increased after the treatment. This phenomenon could, apart from smaller deviations in the analysis results, be related to conversions taking place during the treatments. One result differed radically from all others—the huge decrease (~90%) in concentration of vanillin after treatment with ammonia. The reason for the large concentration decrease in this case is not understood. However, as vanillin is one of the most abundant phenolics in the hydrolysate and the fermentation yields as well as the productivities are similar after treatment with either ammonia or calcium hydroxide, vanillin alone does not contribute significantly to the toxicity of the sample. This agrees with previous findings, as vanillin is not very toxic to *S. cerevisiae* compared to many other lignocellulose-derived aromatic compounds (*8*).

The results suggest that the mechanisms behind the concentration changes of the investigated compounds after the different treatments are complex and deserve further attention in the future. However, there are some well-known reactions that might take place under the conditions used during the treatments and which could possibly account for some of the changes in concentration. For example, aldehydes could undergo nucleophilic addition to the carbonyl group in the presence of ammonia, or aldol-like reactions could occur when the aldehyde is transformed into a reactive nucleophile by formation of its enolate ion under alkaline conditions (*17*). Furthermore, under alkaline conditions phenolic compounds can be transformed into their corresponding phenolate ions, which are known for their high reactivity and undergo further reactions (*17*).

A comparison of the relative concentrations of inhibitors in the hydrolysate before and after detoxification (**Table 3**) showed that treatment with alkali at pH 10.0 was far more effective than was treatment with salt at pH 5.5. The results in **Table 3** also show that it is not important for the concentrations of the compounds whether sulfuric or hydrochloric acid is chosen to adjust the pH to 5.5 after alkaline treatment at pH 10.0. Furthermore, the *F* values obtained in the statistical evaluation were, in general, considerably larger for treatment with alkali than with salt in the hydrolysate. Hence, the results of the one-way ANOVA also imply that alkali treatment at pH 10.0 was more effective than salt treatment at pH 5.5. Only small differences in inhibitor concentrations in the hydrolysate were observed after treatment with salt (samples 9−14 in **Table 3**).

In contrast with furaldehydes and phenolics, aliphatic acids were not removed by the alkali treatments. This is in agreement with previous studies indicating that the concentrations of aliphatic acids in hydrolysates are not much affected by alkali treatments (*9*, *10*, *15*).

According to the results from the HPLC analyses of the filter extracts, <1% of the inhibiting compounds originally determined in the hydrolysate was removed by filtration. However, the compounds that were trapped in the filter were probably adsorbed onto the precipitates formed during the treatments. Hence, the hydrolysates are actually detoxified through alkali treatment, and the decrease in concentration of different inhibitors is most likely due to degradation rather than trapping by the filtration procedure. These results are in agreement with a previous assumption made by Martinez et al. (*9*).

The results of the chemical analyses in this study are in good agreement with the results of the study of overliming of a bagasse hemicellulose hydrolysate conducted by Martinez et al. (*9*). They found that the concentration of furans was dependent on the amount of added calcium hydroxide. The total furan content was reduced by ~55% with optimal overliming conditions, which is considerably more than in this study. However, Martinez et al. conducted their overliming experiment at 60 °C, whereas we performed our experiments at room temperature. Furthermore, consistent with analyses in this study, they could not detect any significant effects of overliming on the concentrations of aliphatic acids in the hydrolysates. However, in contrast with the study performed by Martinez et al. (*9*), where unidentified compounds could have been important for the toxicity of the hydrolysates, we have identified nearly all major peaks present in the HPLC chromatograms.

Chemical Effects of Alkali and Salt Treatment of the Inhibitor Cocktail. The toxic effects of HMF, 2-furaldehyde, acetic acid, and formic acid (*7*) as well as coniferyl aldehyde and ferulic acid (*8*) on *S. cerevisiae* are well-known. The inhibitor cocktail was composed not only to contain representative toxic compounds but also so that it would be possible to monitor the complete chemical composition of the sample by means of HPLC analysis (furaldehydes and phenolics) and HPAEC analysis (aliphatic acids and sugars). According to the analysis of the individual furaldehydes and phenolics present in the cocktail, the concentrations barely changed after any of the treatments, with the exception of 2-furaldehyde and coniferyl aldehyde. Furthermore, it should be noted that the concentration of HMF in some cases increased. This could be explained by the partial degradation of the glucose added to the inhibitor cocktail to form HMF (*18*) or, possibly, by minor variations in the analysis procedure. In contrast to the results obtained for the hydrolysate, the comparison of the relative concentrations of inhibitors showed that treatment with alkali or salt was equally effective in the cocktail. As a consequence, the *F* values obtained in the one-way ANOVA were comparable for treatments with alkali and with salt. The three aromatic compounds (3,4-dihydroxybenzaldehyde, 4-hydroxybenzoic acid, and cinnamic acid) that were detected in the cocktail in addition to the two expected aromatic components, coniferyl aldehyde and ferulic acid, were present in low concentrations (0.4−2.1 ppm) that would not be expected to influence the fermentability (*8*).

Effects of Alkali and Salt Treatment on Sugars and Fermentability. A disadvantage with alkali detoxification is that the total amount of sugars to some extent decreases (**Table 6**). This drawback has previously been addressed by Larsson et al. and Martinez et al. (*9*, *10*, *15*). The inhibitor cocktail was less toxic to the yeast than the hydrolysate, and in agreement with previous studies the fermentability increased after the alkali treatments, both for the hydrolysate and for the inhibitor cocktail.

The productivity was greatly enhanced after treatment with calcium hydroxide or ammonia for the hydrolysate and inhibitor cocktail, correspondingly. It was even higher after these treatments than for the reference solution that contained neither inhibitors nor extra Ca^{2+} or NH_4^+. According to the analysis of the calcium content of the samples after detoxification, the samples that were treated with calcium hydroxide at pH 10.0 contained more calcium than the samples that were treated with calcium sulfate at pH 5.5; thus, the calcium precipitated to a

larger extent in the treatments at pH 5.5, and a slight positive effect may come from the higher calcium concentration. However, the samples treated with calcium sulfate at pH 5.5 did not show very much better fermentability than the untreated samples, so the combination of calcium and high pH is needed to provide the full positive effect of overliming. The results obtained with the inhibitor cocktail indicate that the effects of overliming are difficult to explain solely by considering removal of inhibitors. Ammonia also gave better results than sodium. It is not unlikely that an additional nitrogen source has a positive effect on the subsequent fermentation. Martinez et al. (*9*) used *Escherichia coli* LY01 in the fermentations and, therefore, direct comparisons with regard to the overliming effect on the fermenting organism are difficult to make.

Conclusions. Treatment at pH 10.0 affected the compounds in the hydrolysate more than addition of the corresponding cation at pH 5.5. Furthermore, alkali detoxification of hydrolysates with calcium hydroxide and ammonia resulted in a more efficient decrease in the concentration of furaldehydes than sodium and potassium hydroxide. Whether sulfuric acid or hydrochloric acid was used for acidification to pH 5.5 after treatment at alkaline conditions did not matter with regard to the inhibitor concentrations. Analyses of supercritical fluid extracts of the solid residues formed after alkali treatment of hydrolysates implied that changes in the concentrations of furaldehydes and phenolic compounds were mainly due to chemical conversions rather than to removal by filtration. The effect of different treatments on a cocktail composed of known inhibitors was less extensive and less complex than treatments of the hydrolysate. For both the hydrolysate and inhibitor cocktail, treatments at pH 10.0 resulted in better fermentability than treatments at pH 5.5. Detoxification with calcium hydroxide and ammonia worked better than detoxification with sodium hydroxide. The effects of alkali and salt treatments on the fermentability of the inhibitor cocktail suggest that the mechanisms of alkali detoxification are difficult to explain solely by the removal of inhibitors. The possibility that positive effects of added compounds or of compounds formed at high pH play a role in improving the fermentability therefore deserves future attention.

ABBREVIATIONS USED

ANOVA, analysis of variance; API, atmospheric pressure ionization; CE, capillary electrophoresis; DAD, diode array detector; ESI-MS, electrospray ionization mass spectrometry; FID, flame ionization detector; HMF, 5-hydroxymethyl-2-furaldehyde; HPAEC, high-performance anion exchange chromatography; p.a., pro analysi; PAD, pulsed amperometric detection; SFE, supercritical fluid extraction.

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