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Limits for Alkaline Detoxification of Dilute-Acid Lignocellulose Hydrolysates

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Abstract

In addition to fermentable sugars, dilute-acid hydrolysates of lignocellulose contain compounds that inhibit fermenting microorganisms, such as *Saccharomyces cerevisiae*. Previous results show that phenolic compounds and furan aldehydes, and to some extent aliphatic acids, act as inhibitors during fermentation of dilute-acid hydrolysates of spruce. Treatment of lignocellulose hydrolysates with alkali, usually in the form of overliming to pH 10.0, has been frequently employed as a detoxification method to improve fermentability. A spruce dilute-acid hydrolysate was treated with NaOH in a factorial design experiment, in which the pH was varied between 9.0 and 12.0, the temperature between 5 and 80°C, and the time between 1 and 7 h. Already at pH 9.0, >25% of the glucose was lost when the hydrolysate was treated at 80°C for 1 h. Among the monosaccharides, xylose was degraded faster under alkaline conditions than the hexoses (glucose, mannose, and galactose), which, in turn, were degraded faster than arabinose. The results suggest that alkali treatment of hydrolysates can be performed at temperatures below 30°C at any pH between 9.0 and 12.0 without problems with sugar degradation or formation of inhibiting aliphatic acids. Treatment with $Ca(OH)_2$ instead of NaOH resulted in more substantial degradation of sugars. Under the harsher conditions of the factorial design experiment, the concentrations of furfural and 5-hydroxymethylfurfural decreased while the total phenolic content increased. The latter phenomenon was tentatively attributed to fragmentation of soluble aromatic oligomers in the hydrolysate. Separate phenolic compounds were affected in different ways by the alkaline conditions with

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some compounds showing an increase in concentration while others decreased. In conclusion, the conditions used for detoxification with alkali should be carefully controlled to optimize the positive effects and minimize the degradation of fermentable sugars.

Index Entries: Bioethanol; lignocellulose hydrolysates; fermentation inhibitors; alkali detoxification; sugar degradation.

Introduction

Consumption of fossil fuels in the transportation sector generates carbon dioxide, which may act as a greenhouse gas promoting global warming. Ethanol produced from renewable resources serves as an alternative to fossil fuels and may not result in a net addition of carbon dioxide to the atmosphere. Ethanol can also be used as an additive to gasoline instead of methyl *tert*-butyl ether. Lignocellulose (e.g., forestry wastes), is an abundant and inexpensive raw material considered for production of bioethanol *(1)*. Cellulose and hemicellulose can be degraded to monosaccharides that are subsequently converted to ethanol by microorganisms, such as baker's yeast, *Saccharomyces cerevisiae*.

One problem associated with degradation of lignocellulose polysaccharides to monosaccharides by dilute-acid hydrolysis is the simultaneous formation of compounds that are toxic to the fermenting organism. These inhibitory compounds include furan derivatives, phenolics, and aliphatic acids *(2–11)*. Since the inhibitors decrease the ethanol productivity and consequently make the production of ethanol more expensive, it is desirable to decrease the concentration of the toxic compounds in the hydrolysates prior to fermentation. Various detoxification methods for removal of inhibitors have previously been carefully investigated, such as alkaline treatment at pH values of about 10, enzymatic treatment, and treatment with ion-exchange resins *(5,7–9)*. Overliming—i.e., treatment with $Ca(OH)_2$ at pH values of about 10, and sometimes at elevated temperature—is a widely used method for detoxification of lignocellulose hydrolysates *(5,7,8,10,12)*. However, the center of interest in these investigations has been on fermentability as well as on concentration of inhibitors before and after treatment, and little effort has been made to optimize the parameters during alkali treatment. A possible drawback with alkali detoxification that has so far not been carefully investigated is the concomitant decrease in the concentration of fermentable sugars.

It is well known that sugars degrade in aqueous alkaline solutions *(13–19)*. The parameters that influence the different degradation pathways and rearrangements of sugars is also known. However, most of the investigations *(14–19)* providing this evidence were performed on standard solution mixtures, and therefore, the knowledge gained might not necessarily apply for dilute-acid hydrolysates because of their complex nature. Furthermore, alkali treatment of hydrolysates has proven to be an efficient detoxification method; thus, it would be desirable to perform an investigation to clarify which factors are the most important during the alkali treatment. The main goal would be to find experimental settings in which the detoxification effect is at a maximum and in unison with the degradation of fermentable sugars at a minimum.

In the present study, a factorial design experiment was used to elucidate the limits for alkaline detoxification of a dilute-acid hydrolysate of spruce regarding the individual and synergistic effects of temperature, concentration of hydroxyl ions (pH), and duration of treatment on the concentrations of inhibitors and fermentable sugars. The variables—temperature, pH, and time—were changed simultaneously. Normally, $Ca(OH)_2$ is used in alkali treatments of hydrolysates, so called overliming, but to avoid solubility problems, NaOH was used to raise and maintain the pH, using a pH-control unit. In addition, some experiments were performed with $Ca(OH)_2$ to examine whether sodium and calcium had different effects on the hydrolysate composition at the applied conditions. The concentrations of different monosaccharides were analyzed as well as changes in the concentration of furan aldehydes, aliphatic acids, total phenolics, and selected separate phenolic compounds. Finally, the results were evaluated using response surface modeling.

Materials and Methods

Chemicals

Methanol (p.a.), acetic and formic acid (p.a.), acetonitrile (high-performance liquid chromatography [HPLC] quality), 4-hydro-xybenzoic acid (for synthesis), and vanillin (4-hydroxy-3-methoxy-benzaldehyde) (for synthesis) were supplied by Merck (Darmstadt, Germany). Catechol, furfural, and vanillic acid were from ICN Biomedicals (Aurora, OH). Arabinose (≥99%), galactose (≥99%), glucose (≥99%), mannose (≥99%), xylose (≥99%), and cinnamic acid (≥99%) were from Fluka (Buchs, Switzerland). Coniferyl aldehyde, 5-hydroxymethylfurfural (HMF) (99%), and fucose (≥99%) were purchased from Sigma-Aldrich (Steinheim, Germany). All water used was of Milli-Q quality, i.e., ion exchanged and filtered to yield a low conductivity, here 0.05 μS/cm (Millipore, Bedford, MA).

Preparation of Hydrolysates

A two-step dilute-acid hydrolysate of Norway spruce (*Picea abies*) was used. Chipped Norway spruce was impregnated with H_2SO_4 (0.5% [w/v]) prior to 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 H_2SO_4 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 of the final hydrolysate was 1.9.

Procedure

Optimal conditions for preventing decomposition of sugars during alkali detoxification were investigated using a fractional factorial experimental design with MODDE 4.0 software from Umetrics AB (Umeå, Sweden). The experimental design was used to investigate the influence of pH, temperature, and duration of treatment. To cover the entire area of interest with as few experiments as possible, all variables were changed simultaneously and the influences of the factors on the responses were investigated (n = 17 and degrees of freedom = 11). All variables were given minimum and maximum values based on previous knowledge on the topic. The acidity was varied between pH 9.0 and 12.0, temperature between 5 and 80°C, and time between 1 and 7 h, at the high (H) and low (L) levels with an additional midpoint (M) at pH 10.5, 42.5°C, and 4 h, respectively. The MODDE software (Umetrics AB) generated the plan for the factorial experiment (17 experiments; LLL, HLL, LHL, HHL, LLH, HLH, LHH, HHH, LMM, HMM, MLM, MHM, MML, MMH, and triplicate of MMM to yield the reproducibility).

Portions of the hydrolysate (40 mL) were treated according to the plan. A pH-stat, TIM 900 Titration Manager (Radiometer Analytical A/S, Copenhagen, Denmark) was used in titration mode where the titrator kept a constant pH by continuously adding a titrant. The titrant used was 5 N NaOH. It was necessary to use a pH-stat since degradation of carbohydrates will occur with formation of various organic acids that could alter the pH of the samples during the experiment. The experiments were performed in the presence of air to resemble real-life conditions for detoxification of hydrolysates. After treatment the solution volume was adjusted to 100 mL with Milli-Q water prior to analysis.

To compare the effect of using either NaOH or $Ca(OH)_2$ for the alkali treatments, an additional set of experiments was conducted at six selections of pH, temperature, and reaction time. These settings comprised treatment at pH 10.0 and 25 or 60°C for 1 h, pH 9.0 and 80°C for 1 and 7 h, and pH 10.5 and 42.5°C for 4 and 7 h.

Ultraviolet Absorbance

The absorbance of the hydrolysates (largest peak in the UV spectrum) was measured at 282 nm using an HP 8453 UV-VIS spectrophotometer (Agilent, Palo Alto, CA). The hydrolysates were diluted 1000 times with Milli-Q water prior to determination of absorbance.

Measurement of Total Phenolics

The total amounts of phenolics in the hydrolysates were estimated with a spectrophotometric method based on the Folin & Ciocalteu reagent (Sigma, Steinheim, Germany). The samples were diluted 25 times with Milli-Q water, and 1 mL of the diluted sample was transferred to a 50-mL volumetric flask, to which 3 mL of the Folin & Ciocalteu reagent (2 mol/L) and 30 mL of Milli-Q water were added followed by thorough mixing. After 5–8 min, 10 mL of 20% sodium carbonate solution was added and the volume was adjusted to 50 mL. The mixture was stirred for 2 h, after which the absorbance was measured at 760 nm. The amounts of phenolics were determined from an external calibration curve based on vanillin, since it was the most abundant phenol in the hydrolysate.

HPLC-Analysis of Furans and Phenolics

Analysis of furfural, HMF, catechol, coniferyl aldehyde, 4-hydroxybenzoic acid, vanillic acid, vanillin, and cinnamic acid was performed using an HP 1100 Series HPLC-system consisting of an automatic degasser; a binary pump; an auto-sampler; a UV detector; and an HP 1050 Series diode-array detector, DAD (Hewlett-Packard, Palo Alto, CA). The analytes were separated on 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). A mobile phase gradient, consisting of Milli-Q water and acetonitrile both containing 75 µL/L of formic acid, started with 5% acetonitrile for 5 min, after which the acetonitrile content increased linearly to 10% after 10 min; 30% after 20 min; 50% after 40 min; and, finally, 90% acetonitrile after 50 min. The column was equilibrated with 5% acetonitrile for 10 min before every injection. The injection volume was 5 µL. All analyses were performed as duplicates.

Five point calibration curves for each individual compound were used for quantification. Furthermore, to quantify the furans, the samples had to be diluted 75 times prior to injection. The UV detector was set at 254 nm and the DAD was set at 210, 254, 280, and 330 nm. Wavelengths for quantification were selected for each compound on the basis of absorption maximum for the analyzed compound as well as absorption patterns for coeluting compounds. The furans, furfural and HMF, as well as vanillic acid and cinnamic acid were quantified at 254 nm, coniferyl aldehyde and catechol at 280 nm, 4-hydroxybenzoic acid at 210 nm, and vanillin at 330 nm. Moreover, sum integrations of two retention time windows were performed at 280 nm to obtain rough estimations of the relative furan and phenolic content of the samples. The first retention time window ranged from 0 to 13.5 min and the second from 13.5 to 50 min. The former gave an approximate value of the furan content and the latter an approx value of the phenolic content of the samples.

The analyzed phenolics were chosen owing to their high abundance in the hydrolysates. Together, they account for >90% of the phenolics known to be present in this type of hydrolysate *(12)*.

Analysis of Aliphatic Acids

Analysis of aliphatic acids was performed on a Beckman P/ACE MDQ capillary electrophoresis instrument with a 60 cm × 50 µm id fused silica capillary. All samples were filtered through a 0.45-µm Whatman cellulose acetate filter prior to hydrodynamic injection at 15 psi for 4 s. The voltage was set to 20 kV at reversed polarity. The electrolyte had a pH of 9.8 and was composed of 5.0 m*M* trimellitic acid, 50 m*M* tris(hydroxymethyl)-aminomethane, 1.0 m*M* tetradecyl trimethylammonium bromide, and 0.5 m*M* Ca^{2+}. The electrolyte was filtrated through a 0.2-μm cellulose nitrate filter and degassed using helium before use. Detection was performed 50 cm from the injection site by indirect UV at 220 nm.

Analysis of Sugars

Arabinose, galactose, glucose, mannose, and xylose were determined by high-performance anion-exchange chromatography with a Dionex DX 500 chromatography system coupled to pulsed amperometric detection (Dionex ED 40) using a CarboPac PA-1 column (all from Dionex, Sunnyvale, CA). Initially, the column was activated with a mixture of 200 m*M* NaOH and 70 m*M* sodium acetate for 5 min. The eluent was then changed to Milli-Q water (1 mL/min) and the sample was injected after 7 min. After sample injection, an isocratic elution (1 mL/min) with pure Milli-Q water and post column addition of 300 m*M* NaOH was applied. L-Fucose was used as the internal standard.

Results

Hydrolysate

The absolute concentrations of the individual compounds in the untreated hydrolysate were 21.9 g/L glucose, 3.3 g/L galactose, 16.4 g/L mannose, 1.7 g/L arabinose, 8.5 g/L xylose, 3.1 g/L acetic acid, 0.9 g/L formic acid, 1.1 g/L levulinic acid, 2.0 g/L HMF, 0.5 g/L furfural, 1.9 mg/L catechol, 1.1 mg/L cinnamic acid, 54.0 mg/L coniferyl aldehyde, 39.2 mg/L 4-hydroxybenzoic acid, 17.3 mg/L vanillic acid, and 107.3 mg/L of vanillin. The initial concentration of total phenolics measured by the Folin & Ciocalteu method was 3.1 g/L when vanillin was used as the standard. All results presented in the next section were calculated by the MODDE software, which generated models by a multivariate equation fit. Values for the fit were given as the explained variation (R^2) and the predicted variation (Q^2). All changes in concentrations provided later in the text are given in percent of the initial values. Furthermore, all reported significance values in the text are based on a 95% confidence interval.

Factorial Design Using NaOH

The models had very good fits with R^2 and Q^2 values ranging from 0.8 to 0.95 for the different monosaccharides (arabinose, galactose, glucose, mannose, and xylose). Furthermore, increased temperature and pH had significant effects, temperature being more important, on the degradation of all sugars according to the coefficient plots.

All sugars were degraded already after 1 h at the highest temperature and pH applied. However, more moderate conditions indicated that the degradation of the individual sugars was somewhat more extensive the longer time the treatment lasted (data not shown). Moreover, degradation of all the individual monomeric sugars followed practically the same pattern at the various temperatures and pH values (Fig. 1A). However, xylose had a tendency to degrade slightly more easily than the other sugars at increased temperature and pH, while arabinose tended to be the most resistant (Fig. 1B,C). Furthermore, the individual sugar concentrations seemed to be independent of the pH at low temperatures (5°C), but the pH dependency increased with increased temperature (Fig. 1A). That is, higher pH resulted in more extensive degradation; all sugars totally disappeared at 80°C and pH 12.0, whereas 40–50% disappeared after treatment at 80°C and pH 9.0.

The results on aliphatic acids showed that increased temperature and pH had significant effects, temperature being more important, on the formation of formic acid, but only temperature had a significant effect on the formation of acetic acid and neither temperature nor pH had significant effects on the concentration of levulinic acid according to the coefficient plots (not shown). The model had a very good fit for formic acid, with an R^2 value of 0.92 and a Q^2 value of 0.80, an acceptable fit for acetic acid ($R^2 = 0.68$ and $Q^2 = 0.36$), whereas there was a poor fit for levulinic acid.

Formic acid and acetic acid showed an increase in concentration at elevated temperature and pH according to the response surfaces (Fig. 2A,B). However, there was an approx fourfold increase in formic acid and only a twofold increase in acetic acid at the harshest conditions (80°C and pH 12.0). Furthermore, formic acid and acetic acid were not degraded to any larger extent since the relative concentration never fell below 100% in the response surface plots (Fig. 2A,B).

The concentration changes in levulinic acid were far more moderate than those of acetic and formic acid, that is, the concentration changes were no more than ±20% around the initial value for levulinic acid (Fig. 2C).

Both the temperature and pH were significant for the changes in concentrations of furfural and HMF according to the coefficient plots (not shown). The R^2 and Q^2 values were 0.85 and 0.58 for furfural and 0.81 and 0.48 for HMF. Both furfural and HMF were more easily degraded, probably to acetic and formic acid, when pH was increased from 9.0 to 12.0 and the degradation increased with increasing temperature Fig. 3A,B). Furfural and HMF were absent after 1 h at 80°C and pH 12.0.

Regarding separate phenolic compounds, determined with HPLC, the temperature and pH were only significant ($R^2 = 0.87$ and $Q^2 = 0.68$) for the concentration of vanillic acid. Roughly a threefold increase in the concentration of vanillic acid was observed at 80°C and pH 12.0 (Fig. 3C).

According to the UV measurements at 282 nm both the temperature and pH ($R^2 = 0.87$ and $Q^2 = 0.68$) were significant for the concentration of the compounds that absorbed light at this wavelength. At low temperatures, a decrease in absorptivity from 100 to 70% was observed when the pH was increased from 9.0 to 12.0 (Fig. 4A). When the temperature was increased

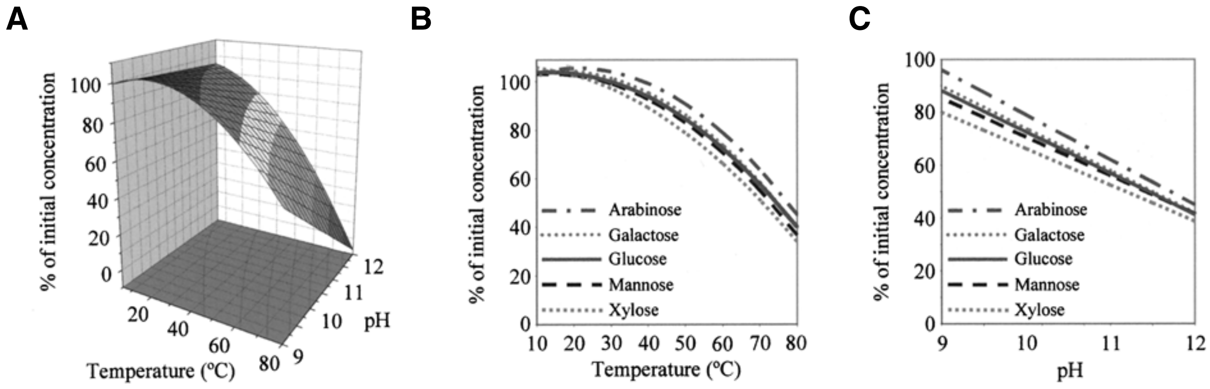

Fig. 1. Response surface (4-h treatment) for glucose **(A)** as well as models for degradation of monosaccharides as a function of increasing temperature at pH 10.0 for 1 h **(B)** and increasing pH at 60°C for 1 h **(C)**.

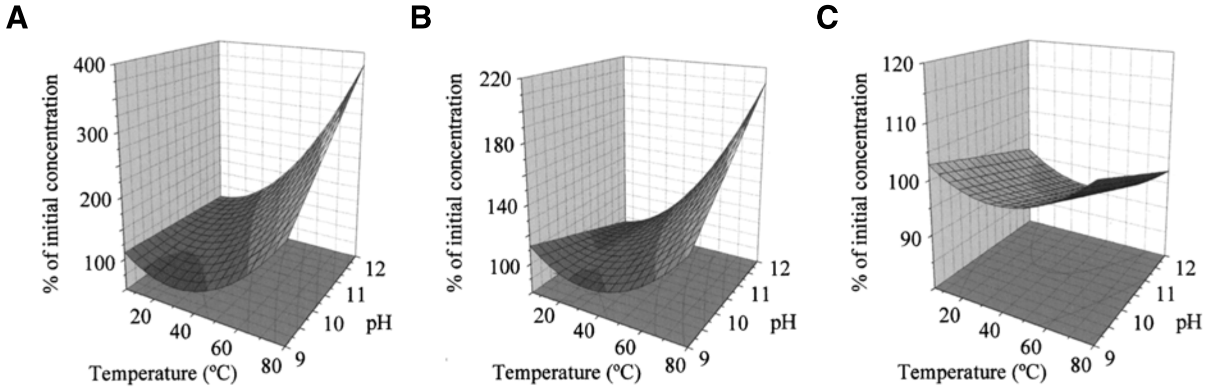

Fig. 2. Response surfaces (4-h treatment) for **(A)** formic, **(B)** acetic, and **(C)** levulinic acid.

from 5°C, there was an initial decrease in absorbance. By contrast, at temperatures above 40°C the absorbance increased drastically at high pH.

The content of phenolic compounds could be estimated by integration of the area in the HPLC chromatogram corresponding to compounds eluting after 13.5 min. High temperature and pH gave an increase in total phenolics (Fig. 4B).

The Folin & Ciocalteu measurements, used to estimate the total concentration of phenolics, largely showed an increase with increased temperature and pH (Fig. 4C). The temperature and pH were significant ($R^2 = 0.94$ and $Q^2 = 0.86$) for the total phenolic content. A fivefold increase in phenolic concentration was seen under the harshest conditions, 80°C and pH 12.0. However, note that a small decrease could be found at 25°C and pH 10.0, i.e., under normal overliming conditions.

Comparison of NaOH and $Ca(OH)_2$

Under mild conditions, no significant degradation of sugars could be determined with either NaOH or $Ca(OH)_2$. At 60°C and pH 10.0, approx 10% of the monosaccharides were lost and a small difference between NaOH and $Ca(OH)_2$ was observed (Table 1). When the conditions were more severe, $Ca(OH)_2$ treatment clearly resulted in more extensive degradation than treatment with NaOH (Table 1).

Discussion

It is well known that sugars can mutarotate under mild alkaline conditions in aqueous solutions. Moreover, it is also known that harsher alkaline conditions catalyze enolization reactions, provided the sugars have an α-hydrogen atom, which, in turn, might lead to isomerization reactions *(13,20)*. Furthermore, β-elimination reactions, leading to the formation of dicarbonyl compounds, might occur simultaneously or subsequent to an enolization reaction *(13,20)*. In time, the β-eliminations will lead to formation of saccharinic acids *(13,20)*. In addition to saccharinic acids, compounds with fewer carbon atoms than the monosaccharides, such as formic acid, are formed. These reactions can occur through retroaldolization as well as splitting or benzilic acid rearrangement of dicarbonyl compounds *(13,14)*.

The degree of degradation or rearrangement of the sugars depends on a variety of factors, such as concentration and type of sugar, temperature, duration of treatment, as well as concentration and type of alkali *(14,16,18–20)*. Because of the high reactivity of ketoses, they appear to be more important as intermediates in degradation of monosaccharides than aldoses. Furthermore, enolization seems to be the rate-limiting step during isomerization and degradation of monosaccharides in aqueous alkaline solutions *(15)*. Higher pH values and the presence of calcium ions increase the enolization and retroaldolization rate *(14,15)*.

The most critical area of question of this study was the possible degradation of fermentable sugars during the treatments. When the temperature

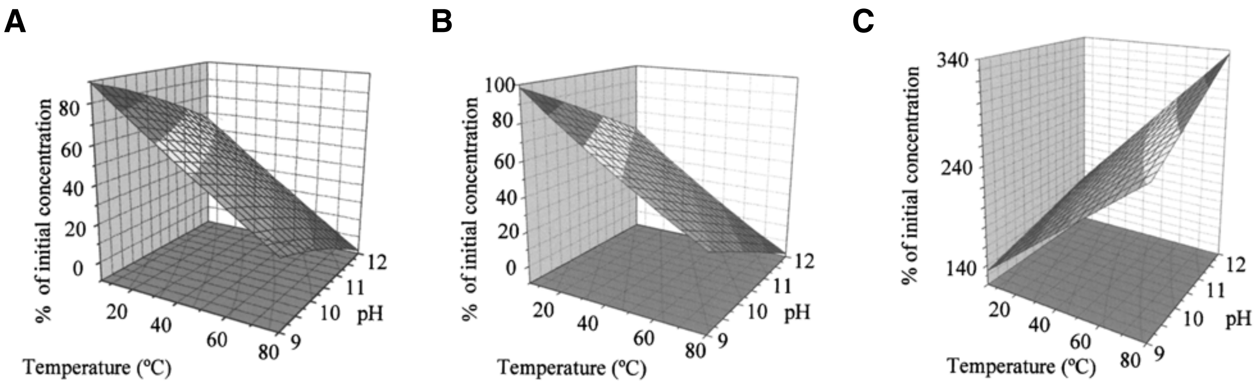

Fig. 3. Response surfaces (4-h treatment) for **(A)** furfural, **(B)** HMF, and **(C)** vanillic acid.

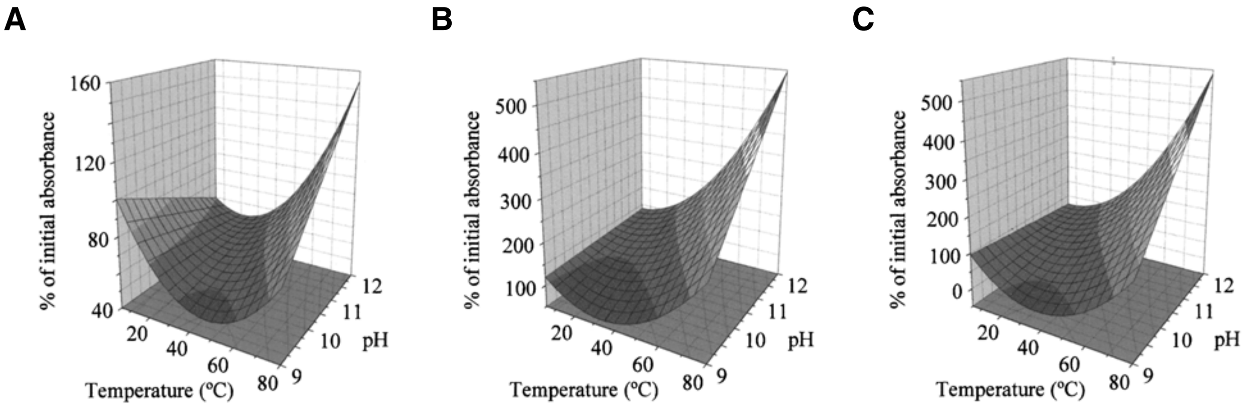

Fig. 4. Response surfaces (4-h treatment) for **(A)** UV absorbance at 282 nm, **(B)** total phenolics determined by HPLC (after 13.5-min elution), and **(C)** total phenolics determined by the Folin & Ciocalteu method.

Table 1
Comparison of Relative Concentrations of Monosaccharides
(Percent Compared to Untreated Hydrolysate)
After Treatment with NaOH or $Ca(OH)_2$ at Various Settings

Alkali	pH	Temperature (°C)	Time (h)	Arabinose	Galactose	Glucose	Mannose	Xylose
NaOH	10.0	25	1	97	97	97	97	98
$Ca(OH)_2$	10.0	25	1	99	99	100	100	100
NaOH	10.0	60	1	92	92	89	87	86
$Ca(OH)_2$	10.0	60	1	88	83	83	81	77
NaOH	9.0	80	1	85	78	76	71	67
$Ca(OH)_2$	9.0	80	1	74	61	60	59	49
NaOH	9.0	80	7	59	42	45	38	20
$Ca(OH)_2$	9.0	80	7	21	8	12	8	4
NaOH	10.5	42.5	4	89	84	80	79	73
$Ca(OH)_2$	10.5	42.5	4	85	83	79	88	78
NaOH	10.5	42.5	7	81	79	75	74	69
$Ca(OH)_2$	10.5	42.5	7	65	46	65	63	42

exceeded 30°C, an extensive degradation of the different sugars started and pH became an important factor in the degradation (Fig. 1). The higher the pH, the more extensive was the degradation. Note that both hexoses and pentoses were degraded similarly (Fig. 1). These results should be compared with the temperature and pH values of previously reported overliming experiments; pH 10.0 at room temperature *(5)*, pH 9.4–10.7 at 25°C or pH 7.5–9.8 at 60°C *(7)*, and pH 10.0–10.5 at 50°C *(10)*. The formation of inhibiting compounds such as aliphatic acids, furan derivatives, and possibly also phenolic compounds during the treatments was also important. According to the response surfaces (Fig. 2), there was a clear increase in acetic and formic acid concentration when the temperature and pH were raised. Because the initial concentration of the sugars was much higher than the concentration of acids, partial sugar degradation resulted in major changes in the concentration of formic and acetic acid. In the case of levulinic acid, the picture was somewhat more complicated. Here, it seems that the rate of breakdown of levulinic acid was equal to the rate of formation owing to breakdown of hexoses. A difference between levulinic acid, on one hand, and formic and acetic acid, on the other, is that levulinic acid has a keto group that could be attacked by hydroxyl ions at higher pH values.

Because the aliphatic acids might act as inhibitors *(4)*, it would be desirable to keep the concentration of these compounds low. The response surfaces (Fig. 2) suggest that this could be accomplished at any pH between 9.0 and 12.0 as long as the temperature does not exceed 30°C. It was clear that a general degradation of furaldehyde and HMF took place at high temperature and pH (Fig. 3A,B).

Forsskåhl et al. *(17)* detected 11 different forms of phenolics after treatment of separate solutions of D-glucose and D-xylose under nitrogen with 0.63 *M* NaOH for 4 h at 96°C. This finding implies that phenolics potentially could be formed in lignocellulose hydrolysates during alkali treatment as a result of degradation and rearrangement of monosaccharides.

The effect of the various treatments on the concentrations of individual phenolics was difficult to interpret since the response surfaces showed a great discrepancy except for the concentration of vanillic acid (Fig. 3C). Nevertheless, there was an obvious formation of vanillic acid at increased temperatures and pH values. Vanillic acid could possibly be formed through oxidation of vanillin, however, the response surface for vanillin did not show any evidence for this. Furthermore, air was present during the treatments, which could cause oxidation of the phenolics. The oxidations could in turn result in further reactions and/or degradation of phenolics. In addition, soluble lignin fragments were probably present in the hydrolysate. These lignin oligomers could most likely be degraded at high pH and cause formation of a variety of phenolic compounds at the applied conditions. gel permeation chromatography analyses have shown that spruce hydrolysates contain soluble oligomeric compounds absorbing UV light (unpublished results).

Different aromatic compounds are known to have very different inhibitory effects on *S. cerevisiae (6)*. Therefore, interconversion between specific phenolic compounds may be of major relevance regarding the combined inhibitory effect of the total phenolic content. The data indicated that treatment under alkaline conditions resulted in a decrease in the concentration of some phenolic compounds, such as coniferyl aldehyde, and an increase in the concentration of others, such as vanillic acid.

Because the response surfaces of separate phenolic compounds generally were hard to evaluate, a sum integration was performed in a retention time window (>13.5 min) in the UV chromatogram (280 nm) corresponding to the retention times where the monomeric phenolics eluted, according to the HPLC analyses. The response surface that resulted from the sum integration showed an increase in total phenolic content with increasing temperature and pH (Fig. 4B).

The Folin & Ciocalteu method showed a very clear increase in phenolic content at high temperature and pH (Fig. 4C). There was a fivefold increase in phenolic concentration at the harshest conditions compared to the initial concentration. However, in agreement with previous results *(5,12)* the formation of phenolics did not start at temperatures below 30°C. Consistent with the results of the Folin & Ciocalteu measurements, the sum integrations at 280 nm showed a fivefold increase in phenolic concentration. It is reasonable to state that the formation of phenolics became essential when the temperature exceeded 30°C. The decrease in total phenolic content observed under milder conditions, approx 25°C and pH 9.0–10.0, is in agreement with previous results *(5,12)*.

UV absorbance is known to be a good tool to obtain approximate values for the furan content of acidic hydrolysates *(21)*. The UV response surface (Fig. 4A), showed an initial decrease in absorptivity when the temperature was raised. However, at temperatures above 40°C the absorptivity increased. On the other hand, the concentrations of furfural and HMF decreased at high temperature (Fig. 3A,B). Hence, the absorbance at 280 nm cannot be solely attributed to furfural and HMF. Consequently, compounds that could account for the increase in absorptivity must have been formed during the treatments. These compounds probably encompassed phenolic compounds with high molar absorptivity around 280 nm since the phenolic retention time window in the HPLC separation (Fig. 4B) showed a clear increase in phenolic concentration at the higher temperatures and pH values, as did the Folin & Ciocalteu measurements (Fig. 4C).

Treatment with $Ca(OH)_2$ resulted in more substantial sugar degradation than NaOH (Table 1). This could probably be attributed to the catalytic effect of calcium ions on the enolization reaction *(14,15)*, which may influence both the kinetics of the following benzilic acid rearrangement and the product pattern.

No analyses of inhibitors were performed after the $Ca(OH)_2$ treatments. However, previous results indicate that sodium and calcium hydroxide have similar effects on the concentration of inhibitors *(5)*.

Conclusion

The sugars in a dilute-acid hydrolysate of spruce treated with NaOH showed no major changes in concentrations between pH 9.0 and 12.0 as long as the temperature did not exceed 30°C. However, at 80°C and pH 12.0 all the sugars were completely degraded. Substituting NaOH for $Ca(OH)_2$ led to more rapid sugar degradation. Along with the degradation of the sugars, furfural and HMF were degraded whereas acetic and formic acid were formed. However, the concentration of levulinic acid did not increase. Separate phenolic compounds were affected differently by treatment with alkali. Severe conditions resulted in an increase in total phenolics, possibly as a combined result of sugar degradation and fragmentation of lignin oligomers. The results suggest suitable limits for the conditions that should be used for treatment with alkali and reveal the effect of the treatment on specific sugars and inhibitory compounds.

Acknowledgments

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