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Critical Conditions for Improved Fermentability During Overliming of Acid Hydrolysates from Spruce

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Abstract

Bioethanol can be produced from wood via acid hydrolysis, but detoxification is needed to achieve good fermentability. Overliming was investigated in a factorial designed experiment, in which pH and temperature were varied. Degradation of inhibitory furan aldehydes was more extensive compared to monosaccharides. Too harsh conditions led to massive degradation of sugars and formation of inhibiting acids and phenols. The ethanol productivity and yield after optimal overliming reached levels exceeding reference fermentations of pure glucose. A novel metric, the balanced ethanol yield, which takes both ethanol production and losses of fermentable sugars into account, was introduced and showed the optimal conditions within the investigated range. The findings allow process technical and economical considerations to govern the choice of conditions for overliming.

Index Entries: Detoxification; ethanol; inhibitors; lignocellulose; optimization; *Saccharomyces cerevisiae*.

Introduction

Fuel ethanol can be produced from low-cost lignocellulose materials, such as wood, agricultural and forest residues, and municipal waste, using microorganisms such as *Saccharomyces cerevisiae (1,2)*. One way to generate fermentable sugars from lignocellulose is to use dilute-acid hydrolysis (H_2SO_4) *(3)*. However, inhibitors are simultaneously formed *(4,5)*. To

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achieve a reasonable fermentability, acid hydrolysates can be detoxified prior to fermentation. Overliming, *i.e.*, treatment with $Ca(OH)_2$, is a widely used and efficient detoxification method *(6–11)*.

Larsson et al. *(8)* compared the effects of 12 different methods for detoxification, including alkali treatment with NaOH and $Ca(OH)_2$, on the chemical composition and fermentability of a dilute-acid hydrolysate of spruce. Overliming was one of the most efficient methods and much more efficient than using NaOH under similar conditions.

Data from studies of bagasse hemicellulose hydrolysates [treated with $Ca(OH)_2$ for 30 min at 60°C] also indicated a decrease in the content of furan aldehydes and phenols, while aliphatic acids remained unaffected *(9)*. Using *Escherichia coli* LY01 as the fermenting microorganism, the fermentability of the bagasse hydrolysates was found to increase with decreasing content of furan aldehydes. On the other hand, an addition of three times the original content of furan aldehydes was needed to restore the initial inhibitory effect of the hydrolysate after overliming, which showed that the decrease in furan concentration was not the principal reason for the improved fermentability. The chemical effects of additions of different types of alkali and salts to a dilute-acid hydrolysate of spruce were studied by Persson et al. *(12)*. The results indicated that chemical conversions of inhibitors during the overliming process were important, rather than removal with the precipitating gypsum.

Nilvebrant et al. *(13)* used NaOH to examine the chemical effects of alkaline treatment of spruce dilute-acid hydrolysates. The results showed that pH played an important role in the decrease in the concentration of different sugars when the temperature exceeded 30°C. Treatment at high temperatures resulted in a substantial decrease in the concentration of glucose. Treatment with $Ca(OH)_2$ instead of NaOH—using the same pH, temperature, and time—resulted in a more extensive degradation of sugars, but the effect on the fermentability was not studied.

Results from studies aimed at optimizing overliming of bagasse hydrolysates at 60°C for 30 min showed that the optimal amount of $Ca(OH)_2$ needed for detoxification varied and was dependent on the concentration of mineral and organic acids originally present in each hydrolysate *(10)*. In another attempt to optimize overliming *(11)*, treatment times up to 170 h, at pH 10–12, were used for overliming of dilute-acid spruce hydrolysates. Surprisingly, after 8 h fermentation with *S. cerevisiae*, almost no ethanol had yet been produced in any of the samples, even after treatments resulting in substantial sugar degradation (up to approx. 70%). It is of great importance to understand the effects of overliming, because this will make the optimization and control easier and provide a possibility to achieve an economically more attractive process design. The current study addressed the question whether it is possible to find optimal conditions that combine substantial removal of inhibitors, a low decrease

in the content of sugars, and high improvement of fermentability during overliming.

Materials and Methods

Hydrolysate

A dilute-acid hydrolysate of chipped Norway spruce, *Picea abies*, was used in all of the experiments. The conditions for the hydrolysis process were the same as previously reported *(14)*. After the hydrolysis, the solid fraction was removed by filtration and the liquid fraction, hereafter referred to as the hydrolysate, had a pH of 1.9. Initial concentrations of fermentable sugars and inhibitory compounds were 17.9 g/L glucose, 14.4 g/L mannose, 6.9 g/L xylose, 3.1 g/L galactose, 1.7 g/L arabinose, 3.0 g/L HMF (5-hydroxylmethylfurfural), 0.6 g/L furfural, 1.0 g/L levulinic acid, 2.5 g/L acetic acid, 0.8 g/L formic acid, and 5.1 g/L phenols (as vanillin equivalents).

Analysis of the Composition of the Hydrolysate

Samples of the hydrolysate, taken before and after the treatments, were analyzed with respect to fermentable monosaccharides and inhibitors. The concentrations of glucose and mannose were determined by anion-exchange chromatography using a DX 500 (Dionex, Sunnyvale, CA) equipped with a CarboPac PA-1 column (Dionex) and conditions as previously described *(13)*.

The furan aldehydes HMF and furfural were determined using an HPLC system consisting of a Waters 2690 separation module, equipped with a binary pump, an auto injector, and a photodiode array detector (PDAD) set at 282 nm. The separation was performed on an ODS-AL column (50 × 3 mm, 120 Å, and 5-μm particles) (Waters Milford, MA). The conditions were as previously described *(15)*. The total concentration of phenols was analyzed by a modified spectrophotometric method *(13)* based on the Folin and Ciocalteu's reagent and given as vanillin equivalents.

A Beckman P/ACE MDQ capillary electrophoresis instrument, equipped with a 60 cm × 50 μm ID fused silica capillary (Beckman Coulter, Fullerton, CA), was used as previously described for the analysis of aliphatic acids *(15)*. The samples were filtered through a 0.45 μm cellulose acetate filter (Whatman, Maidstone, UK).

Detoxification

The effect of pH and temperature on detoxification by overliming was investigated in a multilevel factorial experimental design using MODDE 7.0 software (Umetrics AB, Umeå, Sweden). The two variables investigated, pH and temperature, were given five and four levels, respec-

tively, and the treatments were performed during 3 h. All variables were simultaneously changed, creating a full factorial experiment consisting of 24 different samples. The pH and temperature levels were selected on the basis of previous studies *(13,16)* and ranged from pH 8 to 12 (determined at room temperature) and from 5 to 80°C. The experiment at pH 12 and 80°C was excluded because of the limited solubility of calcium hydroxide. Because the fermentability after treatment at pH 9 and 30°C was poor and the chemical changes were minor, two experiments at pH 8 (5°C and 30°C) were excluded.

The detoxifications were performed using a TIM 900 Titration Manager (Radiometer Analytical, Copenhagen, Denmark) in pH-static mode. The pH-meter was calibrated at 25°C. The pH is strongly influenced by temperature and a lower activity is determined by the pH-meter at higher temperatures than at room temperature. A correction function was therefore used to estimate the accurate pH values. The correction function was obtained by rapidly heating a hydrolysate sample that was adjusted to pH 9, 10 or 11 at room temperature. The pH reading was recorded every 10°C up to 80°C. The correction function was calculated as a mean value of the trials. For comparison, two experiments in which sodium hydroxide was used instead of calcium hydroxide were performed at 30°C and were referred to as pH 10 and 11, respectively. After the different treatments, the pH was decreased to 5.5 by addition of hydrochloric acid. The dilution of the hydrolysate, by adjustment of pH, was 5% in all fermented hydrolysates. The samples were filtered before fermentation, using a Büchner funnel.

Yeast Strain and Growth Conditions

Baker's yeast, *Saccharomyces cerevisiae*, obtained from Jästbolaget AB (Rotebro, Sweden) was used in the fermentation experiments. The strain was maintained on agar plates with YEPD medium (2% yeast extract, 1% peptone, 2% D-glucose, 2% agar). Inoculum cultures were grown aerobically in 2000-mL cotton-plugged-conical flasks containing 500 mL YEPD medium on a rotary shaker at 30°C. Cells were harvested in the exponential growth phase by centrifugation (1500*g*, 5 min, and 4°C) and washed with a NaCl solution (9 g/L).

Fermentations

Fermentations were performed during oxygen-limited conditions in 55-mL glass vessels sealed with rubber stoppers. The working volume was 50 mL, of which 47.5 mL was hydrolysate (or alternatively, a glucose solution for the reference fermentation). The hydrolysates were supplemented with nutrients to a final concentration 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. Fermentation of synthetic medium containing 30 g/L glucose and

nutrients was performed as reference. The initial cell concentration was 2 g/L (dry weight) and incubation was performed at 30°C for 30 h with magnetic stirring. Samples (200 μL) were taken and stored at −20°C for analysis.

Analysis of Fermentation Products

Samples taken from the broth were diluted with distilled water and filtered through 0.45 μm GHP Acrodisc syringe filters (Pall Gelman Laboratory, Ann Arbor, MI). The glucose and mannose concentrations in the samples were determined. The ethanol concentration was measured using an HP 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a BP-20 column with a film thickness of 1.0 μm (SGE, Austin, TX) and a flame ionization detector. The conditions were as previously described *(15)*.

The biomass concentration was determined from dry-weight measurements. Duplicate samples (3 mL), taken from the broth at the beginning and the end of the fermentations, were filtered through a 0.45 μm pre-dried and pre-weighed MF-membrane filter (type HA) (Millipore, Milford, USA). The cells were washed with three volumes of distilled water and dried in a microwave oven (Husqvarna Micronett, Sweden) set at a power scale of 3. The filters were then stored in a desiccator before weighing.

Assessment of Fermentability

The maximum mean volumetric productivity of ethanol ($Q_{EtOHmax}$) of the reference fermentation in each series was calculated. The volumetric productivity (Q_{EtOH}) of the hydrolysate-containing samples was calculated at the time when the reference fermentation reached its maximum and expressed as percent of $Q_{EtOHmax}$.

The ethanol yield (Y_{EtOH}) was calculated from data from samples collected after 6 h of fermentation. Y_{EtOH} was defined as the amount of ethanol produced in the fermentation, divided by the amount of glucose and mannose consumed during the same period of time. The ethanol yield of the hydrolysate-containing samples was also calculated as percentage of the reference fermentation.

A new dimension, balanced ethanol yield (Ψ_{EtOH}), was introduced to make a combined assessment of the effect of the detoxification treatment on both the concentration of fermentable sugars and the fermentability. This dimension was calculated as the amount of ethanol produced divided by the total amount of fermentable sugars before the detoxification treatment, i.e., (g EtOH)/(g Σ glucose + mannose), and given as percentage of the reference fermentation. The balanced ethanol yield, ψ_{EtOH}, can be used to draw a 3D-plot from which the optimal overliming conditions may be predicted.

Results

The effect of the different treatments on the concentrations of fermentation inhibitors and fermentable sugars are shown in Table 1. These data from the factorial designed experiments were used to generate graphical models by the MODDE software for changes in the concentrations of sugars, furan aldehydes, acids, and phenols (Figs. 1A–F). Under harsh conditions, there was a clear increase in the concentrations of formic and acetic acid (Table 1). Values for the fit of the models were given as the expected variation (R^2) and predicted variation (Q^2). The model for changes in the concentration of formic acid is displayed in Fig. 1F ($R^2 = 0.84$ and $Q^2 = 0.62$). The shape of the model for acetic acid (not shown) looked essentially the same as that for formic acid. Treatments performed at 5°C had little or no effect on any of the aliphatic acids, regardless of pH.

For the sample called 55°C and pH 11, or at even harsher conditions, the furan aldehydes were almost quantitatively degraded (Table 1). HMF was degraded more rapidly than furfural (Figs. 1C,D, $R^2 = 0.90$, $Q^2 = 0.79$ and $R^2 = 0.89$ and $Q^2 = 0.76$, respectively). Under most of the conditions studied, there was a decrease in the content of phenols (Table 1). However, under harsh conditions there was an increase. This resulted in a saddle-shaped model, which shows that phenols are best removed under intermediate conditions (Figs. 1E, $R^2 = 0.92$ and $Q^2 = 0.80$).

Glucose and mannose were similarly affected (Table 1). Treatment under the harshest conditions resulted in extensive sugar degradation. In the intervals studied, the sugars were similarly affected by changes in temperature and pH (Figs. 1A,B, $R^2 = 0.92$, $Q^2 = 0.82$ and $R^2 = 0.91$ and Q^2=0.81, respectively). The sugars were less affected than the furan aldehydes by overliming (Table 1, Figs. 1A–D). Notably, a plateau could be observed under mild conditions in the models for glucose and mannose (Figs. 1A,B). A corresponding plateau was not present in the models for the furan aldehydes (Figs. 1C,D). The concentration of formic acid did not change very much under milder conditions (Fig. 1F). The conditions that lead to a sharp increase in the concentration of formic acid corresponded with the conditions that lead to a sharp decrease in sugar concentration.

The volumetric ethanol productivity (Q_{EtOH}) for the untreated hydrolysate was only 6% of the value for the reference fermentation (Table 1). Fermentation of the hydrolysates showed that Q_{EtOH} increased after all treatments analyzed, although for the sample pH 9 and 5°C there was a very small improvement. The highest volumetric productivities, which were somewhat higher than for the reference fermentation (reaching as high as 120% for the sample pH 11 and 30°C), were achieved after treatments at high pH and intermediate temperature or intermediate or low pH at high temperatures. Treatment at very harsh conditions clearly resulted in a lower productivity than intermediate conditions, but there was still a considerable improvement compared with untreated

Table 1
Concentrations of Inhibitors and Sugars after Overliming and Assessment of Fermentability of the Alkali-Treated Hydrolysates

Treatment			Inhibitors and sugars[a]								Assessment of fermentability		
			Aliphatic acids			Furan aldehydes			Sugars				
pH	pH_{corr}[b]	Temp (°C)	Formic	Acetic	Levulinic	HMF	Furfural	Phenols	Glu	Man	Q_{EtOH} (% of $Q_{EtOHmax}$)	Y_{EtOH} (% of reference fermentation)	Ψ_{EtOH}[c] (% of reference fermentation)
9	8.9	5	113	104	97	89	100	86	98	98	8	39	8
10	9.9	5	114	104	107	84	99	72	94	96	10	62	10
11	10.9	5	116	104	81	76	92	74	89	88	16	68	15
12	11.9	5	111	105	85	54	58	91	95	95	23	130	28
9	9.1	30	104	111	85	82	96	74	92	92	14	77	14
10	10.1	30	135	114	87	66	80	71	91	91	24	69	23
11	11.1	30	143	112	86	35	47	78	85	87	120	140	120
12	12.1	30	124	109	90	9	5	100	75	76	73	140	87
10.5	10.8	42.5	121	108	92	52	45	98	96	96	55	110	66
8	8.5	55	122	104	101	94	82	88	99	100	22	130	27
9	9.5	55	152	111	86	63	74	58	84	88	110	130	110
10	10.5	55	169	126	100	18	25	78	74	82	110	130	100
11	11.5	55	316	133	80	1	3	125	36	37	54	140	52
12	12.5	55	270	219	122	<1	<1	201	1	1	—	—	—
8	9.1	80	126	183	108	62	47	104	83	88	81	130	98
9	10.2	80	192	229	127	10	4	154	45	47	48	130	58
10	11.2	80	198	182	91	1	<1	249	32	31	—	—	—
11	12.2	80	367	222	118	<1	<1	335	3	2	—	—	—
10	10.1	30	117	121	79	66	81	81	89	97	22	55	21
10	10.1	30	122	125	82	67	85	69	89	95	22	51	21
10[d]	10.1	30	71	110	89	79	95	80	94	97	17	53	16
10[e]	10.1	30	107	128	102	77	91	87	89	92	18	61	17
11[e]	11.1	30	105	98	84	56	53	94	92	96	54	130	65
Untreated									6	21	5		

[a] The concentrations are indicated in percent of the initial values in the untreated hydrolysate.
[b] Because pH varies with temperature, the corrected values were calculated.
[c] Balanced ethanol yield.
[d] Treated for 1 h, all other samples treated for 3 h.
[e] Treatment with NaOH.

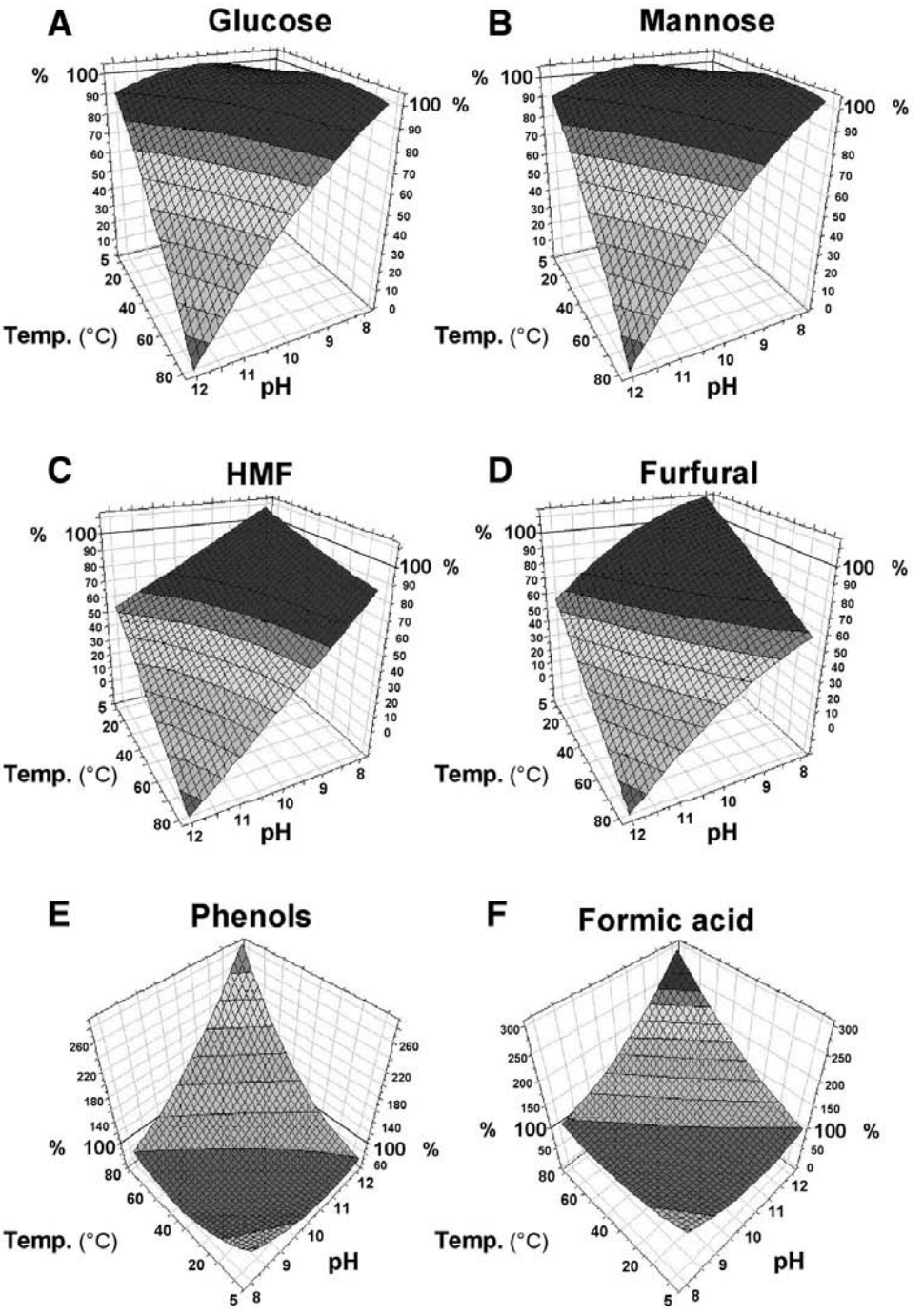

Fig. 1. (A–F) Models of the effects on the concentrations of fermentable sugars and inhibitors during overliming. Observe the reversed axes for phenols **(E)** and formic acid **(F)**. The temperature-corrected pH values (cf. Table 1) are used in the graph.

hydrolysate. It should also be kept in mind that the initial sugar concentration of the hydrolysate decreased when harsh conditions were used. Only modest improvements in productivity were achieved at 5°C. On the other hand, major improvements in fermentability were possible to achieve at low pH, if very high temperatures were used, as shown by the fact that the sample pH 8 and 80°C reached 81% of the productivity of the reference fermentation. As could be expected, a decrease in the duration time from 3 to 1 h for the sample pH 10 and 30°C resulted in a poorer ethanol productivity. Furthermore, when NaOH was used instead of $Ca(OH)_2$, the improvement in productivity was lower, which was particularly apparent for the sample pH 11 and 30°C (Table 1).

The ethanol yield Y_{EtOH}, in the untreated hydrolysate was 0.08 g/g, which corresponded to 21% of the value obtained for the reference fermentation (Table 1). With respect to Y_{EtOH}, samples treated under harsh conditions, such as sample pH 11 and 55°C, were comparable or even better than those showing the highest ethanol productivity. Notably, for all samples displaying high Y_{EtOH}, the fermentable sugars were essentially consumed within the time period used for the calculation. The fermentable sugars in the samples displaying intermediate Y_{EtOH} were consumed later and the poorest yields were obtained for the sample treated under the mildest conditions and the untreated sample.

The balanced ethanol yield (Ψ_{EtOH}) was high for the samples that also showed high productivity (Table 1). The sample pH 11 and 30°C reached the highest Ψ_{EtOH} (0.44 g of ethanol per g glucose and mannose present before the treatment). Although the fermentation of samples treated under very harsh conditions (samples pH 11 and 55°C; pH 9 and 80°C) was essentially complete at the time when Ψ_{EtOH} was measured and, although the Y_{EtOH} values were high, they never reached a Ψ_{EtOH} higher than 0.19 g/g. This is explained by the substantial sugar degradation (<50% remaining) taking place under these conditions and the result suggests that the Ψ_{EtOH} value is an interesting tool in the evaluation of the overliming data.

The graph of the balanced ethanol yield (Fig. 2) indicates the optimal conditions for overliming. The optimal conditions correspond to a ridge that stretches from low pH and high temperature to high pH and intermediate temperature.

Discussion

Detoxification is necessary to achieve an efficient fermentability of dilute-acid hydrolysates of spruce to ethanol. Overliming is one of the most efficient detoxification methods. However, harsh alkaline conditions reduce the amount of fermentable sugars. The goal with the treatment is to obtain an excellent fermentability, with a minimum loss of fermentable sugars.

The detoxification treatment affected both the concentrations of fermentable sugars and the concentrations of different inhibitors. The

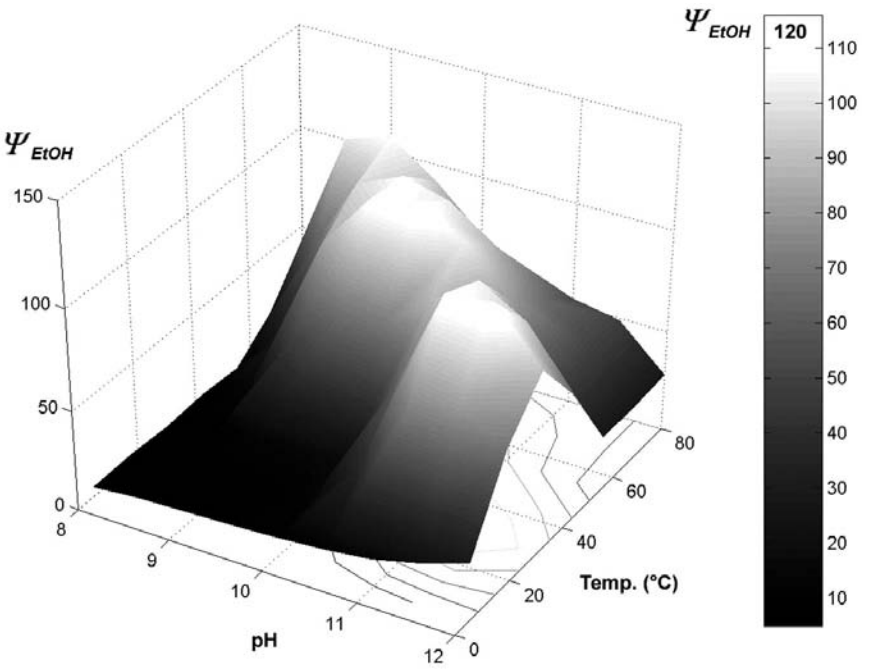

Fig. 2. Model of balanced ethanol yield after overliming of a dilute-acid hydrolysate of spruce. The optimal conditions (temperature-corrected pH values) for overliming are visualized as a ridge that goes from low pH and high temperature to high pH and intermediate temperature.

degradation of sugars was strongly dependent on the conditions. Previous reported data *(13,16)* show a 10% decrease in the concentration of fermentable sugars with NaOH, while the decrease with $Ca(OH)_2$ under the same conditions was as high as 20%. An explanation could be that calcium ions catalyze alkaline degradation of monosaccharides like glucose and mannose, by interaction with their enolate intermediates *(17)*.

The concentration profiles for the different inhibitory compounds showed similar trends after treatment with $Ca(OH)_2$ (this study) as after treatment with NaOH *(13)*. There were major increases in the concentrations of acetic and, particularly, formic acid under harsh conditions. The high increase observed for formic acid can be related to the simultaneous decrease in the concentrations of sugars, as indicated by the edge of the plateaus in the models for sugars and formic acid (Figs. 1A,B,F). Formic acid is a direct product of alkaline degradation of monosaccharides and so is acetic acid, although via a more complicated route *(18)*.

The furan aldehydes were degraded at alkaline conditions and the removal of furfural was more efficient at higher temperatures. The higher volatility of furfural compared with HMF can be seen in Figs. 1C,D, especially at the lower pH values. An important aspect that was observed

with both NaOH *(13)* and $Ca(OH)_2$ (this work) is that there was very low sugar degradation under mild conditions, while there was a clear decrease in the concentration of HMF. This suggests that conditions that permit a decrease in HMF but not in fermentable sugars can be obtained.

The aldehydes, i.e., both the furan aldehydes and the monosaccharides, are reactive under alkaline conditions. The monosaccharides are present in their cyclic hemiacetal form and expose their aldehyde function during their mutarotation. The monosaccharides present in the hydrolysate possess an acidic hydrogen and form the corresponding enolate when the surrounding conditions are alkaline enough. This enolization is the key stage to the alkali-induced sugar degradation and the formation of, for example, formic acid *(18)*. Because the aldehydes HMF and furfural do not hold any acidic alpha-hydrogen, they cannot form the corresponding enolates. However, the furan aldehydes may take part in base-catalyzed condensations, e.g., aldol reactions with the enolates presented by the monosaccharides at strong enough alkaline conditions *(19)*. It is reasonable to suggest that the degradation of inhibiting furan aldehydes is dependent on the presence of ionized monosaccharides. Thus, a loss of sugars seems inevitable for the efficient removal of furan aldehydes in a mixed aldol reaction. Fortunately, the concentration of monosaccharides is high, as compared to the amount of furan aldehydes present in the hydrolysate and xylose is the easiest to ionize *(13)*.

Phenols are more easily oxidized when present in their corresponding ionized form. Generally, phenol groups are ionized around a pH of 10 (25°C). The concentration of phenols decreased at most conditions tested. However, at alkaline conditions when the phenol groups in dissolved lignin fragments become ionized, this may lead to lignin degradation and formation of new phenols. These probably originate from soluble oligomeric lignin fragments present in the hydrolysate *(13)*.

Traditional overliming conditions or treatment with NaOH (room temperature and pH 10) degraded the initial content of furan aldehydes and the total amount of phenols by approx. 20%, while the content of aliphatic acids and fermentable sugars remained essentially unchanged *(8)*. The overliming resulted in a productivity that was 83% of the reference, which was about twice as high compared to when NaOH was used. These results suggest that overliming can be optimized to achieve a fermentability that is fairly close to what can be observed for a synthetic sugar solution and with a sugar loss below 10%.

In a study on optimization the amount of $Ca(OH)_2$ needed to detoxify bagasse hydrolysates at 60°C for 30 min Martinez et al. *(10)* showed that the optimal addition varied and was dependent on the concentration of mineral and organic acids originally present in each hydrolysate. The highest ethanol concentrations were achieved after treatments at around pH 9, the content of furan aldehydes decreased by approx 50%, and the content of phenols decreased by approx 40%. However, these conditions also resulted in a decrease in the content of sugars by approx 9%. Higher

pH resulted in a more extensive sugar degradation (approx 30% decrease at pH 10) and lower final ethanol concentrations after fermentation. It is evident from different studies on overliming that degradation of sugars is a potential disadvantage *(9,10,11)*. The key to optimization of alkaline treatment is to find conditions that decrease the concentrations of inhibitory compounds, keep the levels of fermentable sugars high, and provide good fermentability, as judged by comparison with a reference fermentation containing only glucose.

Fermentation of all treated hydrolysates resulted in an increase in ethanol productivity, as compared to untreated hydrolysate (Table 1). However, all of the conditions that increase the productivity are not optimal for detoxification. Among the samples that showed high ethanol productivity, there were some in which the alkali treatment resulted in a dramatic decrease in the concentration of fermentable sugars. For economical reasons, these conditions, i.e., high temperature in combination with high pH, should be avoided.

Some of the samples displayed higher ethanol yield than the reference fermentation (Table 1). Previous results indicate that concentrations of aliphatic acids below approx 100 m*M* lead to an increase rather than a decrease in the ethanol yield *(4)*. Results from other studies have shown that acetic acid contributes to high ethanol yield at the expense of biomass and glycerol yields *(20)*. In this study, the initial concentrations of formic, acetic, and levulinic acids in the untreated hydrolysate were 16 m*M* (0.74 g/L), 42 m*M* (2.5 g/L) and 8 m*M* (0.93 g/L), respectively. The total concentration of the three aliphatic acids was thus only 66 m*M*. The samples that showed high Y_{EtOH} had a high total content of aliphatic acids (Table 1). For example, the sample referred to as pH 11 and 55°C contained 114 m*M* aliphatic acids and displayed a Y_{EtOH} that was about 140% of that of the reference fermentation. The samples with moderate ethanol yields had a concentration of aliphatic acids that was lower than 80 m*M*. The results suggest that moderate concentrations of organic acids, such as those found in dilute-acid hydrolysates of spruce, contribute to high ethanol yields rather than causing an inhibition problem. The generation of aliphatic acids during the overliming process thus contributes to high ethanol yields, provided that the concentration in the untreated hydrolysate is not excessive. Weak acids may be responsible for a decrease in the biomass yield and an increase in the ethanol yield by a diversion of ATP from anabolism to consumption by a H^+-pumping ATPase *(21)*.

The balanced ethanol yield Ψ_{EtOH} takes both the fermentability and the sugar degradation into account and permits a meaningful analysis of optimal conditions for overliming (Fig. 2), because a sample with extensive sugar degradation but high productivity would always display a poor Ψ_{EtOH}. For example, the sample called pH 11 and 30°C displayed an ethanol productivity that was 20 times higher than that

observed for untreated hydrolysate and that was even better (120%) than in the reference fermentation, which did not contain inhibitors. Fourteen percent of the fermentable sugars were lost. The balanced ethanol yield was still as high as 0.44 g/g in that sample. If the maximum theoretical ethanol yield is assumed to be 0.51 g/g, the difference is exactly 14% and can thus be accounted for by sugar degradation.

The results showed that overliming at a relatively low pH can be used in combination with a high temperature to achieve very good fermentability. This may be of practical interest considering the fact that the hydrolysate is already heated after the acid hydrolysis process and that less $Ca(OH)_2$ would be needed for the treatment. Alternatively, the temperature can be varied and optimal conditions for overliming can nevertheless be achieved. The approach selected to perform overliming can therefore be governed by economical and process technological considerations.

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References

1. Wheals, A. E., Basso, L. C., Alves, D. M. G., and Amorim, H. V. (1999), *TIBTECH* **17**, 482–487.
2. Galbe, M. and Zacchi, G. (2002), *Appl. Microbiol. Biotechnol.* **59**, 618–628.
3. Lee, Y. Y., Iyer, P., and Torget, R. W. (1999), *Adv. Biochem. Eng./Biotechnol.* **65**, 93–115.
4. Larsson, S., Palmqvist, E., Hahn-Hägerdal, B., et al. (1999), *Enzyme Microb. Technol.* **24**, 151–159.
5. Palmqvist, E. and Hahn-Hägerdal, B. (2000), *Biores. Technol.* **74**, 25–33.
6. Van Zyl, C., Prior, B. A., and Du Preez, J. C. (1988), *Appl. Biochem. Biotechnol.* **17,** 357–369.
7. Barbosa, M. d. F. S., Beck, M. J., Fein, J. E., Potts, D., and Ingram, L. O. (1992), *Appl. Environ. Microb.* **58**, 1382–1384.
8. Larsson, S., Reimann, A., Nilvebrant, N.-O., and Jönsson, L. J. (1999), *Appl. Biochem. Biotechnol.* **77–79**, 91–103.
9. Martinez, A., Rodriguez, M. E., York, S. W., Preston, J. F., and Ingram, L. O. (2000), *Biotechnol. Bioeng.* **69**, 526–536.
10. Martinez, A., Rodriguez, M. E., Wells, M. L., York, S. W., Preston, J. F., and Ingram, L. O. (2001), *Biotechnol. Prog.* **17**, 287–293.
11. Millati, R., Niklasson, C., and Taherzadeh, M. J. (2002), *Process Biochem.* **38**, 515–522.
12. Persson, P., Andersson, J., Gorton , L., Larsson, S., Nilvebrant, N.-O., and Jönsson, L. J. (2002), *J. Agr. Food Chem.* **50**, 5318–5325.
13. Nilvebrant, N.-O., Persson, P., Reimann, A., de Sousa, F., and Jönsson, L. J. (2003), *Appl. Biochem. Biotechnol.* **105–108**, 615–628.
14. Nilvebrant, N.-O., Reimann, A., Larsson, S., and Jönsson, L. J. (2001), *Appl. Biochem. Biotechnol.* **91–93**, 35–49.
15. Sárvári Horváth, I., Sjöde, A., Nilvebrant, N.-O., Zagorodni, A., and Jönsson, L. J. (2004), *Appl. Biochem. Biotechnol.* **114**, 525–538.

16. Sjöde, A. (2003), Master thesis, Royal Institute of Technology, Stockholm, Sweden.
17. de Bruijn, J. M., Kieboom, A. P. G., and van Bekkum, H. (1987), *Recl. Trav. Chim. Pays-Bas* **106**, 35–43.
18. de Bruijn, J. M., Kieboom, A. P. G., van Bekkum, H., and van der Poel, P. W. (1986), *Sugar Technol. Rev.* **13**, 21–52.
19. Pine, S. H., Hendrickson, J. B., Cram, D. J., and Hammond, G. S. (1980), In *Organic Chemistry*, 4th ed. McGraw-Hill NY, pp. 276–278.
20. Taherzadeh, M. J., Niklasson, C., and Lidén, G. (1997), *Chem. Eng. Sci.* **52**, 2653–2659.
21. Henriques, M., Quintas, C., and Loureiro-Dias, M. C. (1997), *Microbiology* **143**, 1877–1883.