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Optimal Conditions for Alkaline Detoxification of Dilute-Acid Lignocellulose Hydrolysates

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Abstract

Alkaline detoxification strongly improves the fermentability of dilute-acid hydrolysates in the production of bioethanol from lignocellulose with *Saccharomyces cerevisiae*. New experiments were performed with NH_4OH and NaOH to define optimal conditions for detoxification and make a comparison with $Ca(OH)_2$ treatment feasible. As too harsh conditions lead to sugar degradation, the detoxification treatments were evaluated through the balanced ethanol yield, which takes both the ethanol production and the loss of fermentable sugars into account. The optimization treatments were performed as factorial experiments with 3-h duration and varying pH and temperature. Optimal conditions were found roughly in an area around pH 9.0/60°C for NH_4OH treatment and in a narrow area stretching from pH 9.0/80°C to pH 12.0/30°C for NaOH treatment. By optimizing treatment with NH_4OH, NaOH, and $Ca(OH)_2$, it was possible to find conditions that resulted in a fermentability that was equal or better than that of a reference fermentation of a synthetic sugar solution without inhibitors, regardless of the type of alkali used. The considerable difference in the amount of precipitate generated after treatment with different types of alkali appears critical for industrial implementation.

Index Entries: Ethanol; lignocellulose; detoxification; alkali; inhibitor.

Introduction

In the production of fuel ethanol by fermentation of dilute-acid hydrolysates, fermentation inhibitors such as furan aldehydes, phenols, and aliphatic acids can cause a considerable decrease in fermentability. Treatment of the hydrolysate with $Ca(OH)_2$ prior to the fermentation, overliming, is a well established method to improve the fermentability. It is one of the most efficient detoxification methods known *(1)* and it has been in the focus of attention of several studies *(2–6)*. Optimization of the

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conditions for overliming of a dilute-acid hydrolysate of spruce has shown that an ethanol productivity and an ethanol yield equal to or even superior to that of a reference fermentation of a synthetic sugar solution can be achieved *(6)*.

One drawback with overliming is the formation of a calcium sulfate precipitate (gypsum). Another drawback is that if the treatment is done under too harsh conditions (high pH and temperature), a considerable degradation of fermentable sugars occurs *(3,5,7)*. Chemical analysis of overliming combined with fermentation experiments suggest that it is difficult to find conditions that separate degradation of fermentable sugars from degradation of inhibiting furan aldehydes *(6)*. Even though very harsh conditions lead to quantitative degradation of some inhibitors, such as furan aldehydes, the fermentability does not increase further, which can be attributed to formation of other inhibitors, such as phenols and formic acid *(6)*. Thus, the treatment has to be optimized to meet the objectives of combining the highest improvement in fermentability with the lowest sugar degradation. A tool for optimizing detoxification procedures is the balanced ethanol yield (ψ_{EtOH}), which takes both the ethanol yield in relation to a reference fermentation of a synthetic sugar solution and the sugar degradation into account *(6)*. Although the problem with sugar degradation can be minimized using this approach, the problem with gypsum formation persists. Other attempts to optimize overliming include the use of titration with NaOH to predict optimal lime addition for detoxification of sugarcane bagasse hydrolysates *(3)*.

A potential approach to overcome the problems associated with overliming is to use another form of alkali. Ammonium does not form poorly soluble salts and previous results suggest that treatment with ammonium hydroxide compares favorable with overliming *(4,8)*. Sodium hydroxide would be another option, but in comparisons performed under similar conditions sodium hydroxide treatment has so far been less efficient than overliming (*see* e.g., refs. *1* and *8)*. Novel studies of overliming *(6)* suggest that the conditions for the treatments would need to be carefully optimized to make comparisons been different forms of alkali truly meaningful. To address this and to find an alternative to overliming, factorial designed experiments for treatment of a dilute-acid hydrolysate with ammonium hydroxide and sodium hydroxide were performed to find the optimal treatment conditions. In order to further clarify the role of ammonia in alkaline treatments and to investigate the importance of pH adjustment during detoxification, an experimental series was carried out in which a combination of ammonium hydroxide and sodium hydroxide was used. In addition, a simultaneous comparison among treatments with calcium hydroxide, ammonium hydroxide and sodium hydroxide was carried out on basis of the results of the optimizations. The benefits and drawbacks of each of the methods are discussed.

Methods

Dilute-Acid Hydrolysate From Spruce

The dilute-acid hydrolysate used was prepared for the Swedish Ethanol Research Program and supplied by Dr. R. Eklund (Midsweden University, Örnsköldsvik, Sweden). The hydrolysate was prepared by a two-step hydrolysis in a 250-L batch reactor. Chipped Norway spruce (*Picea abies)* was impregnated with H_2SO_4 (0.5% w/v). Steam was loaded to 12×10^5 Pa (190°C) and the pressure was kept for 10 min. The liquid and solid fractions were separated by filtration. The solid fraction was washed with water and reimpregnated with H_2SO_4 and loaded into the reactor again. Steam was added to 21×10^5 Pa (215°C) and the pressure was kept for 10 min. The liquid fraction was recovered by filtration and pooled with the liquid fraction from the first step. This solution, referred to as the hydrolysate, had a pH of 1.9. The hydrolysate contained 17.3 g/L glucose, 13.3 g/L mannose, 3.1 g/L 5-hydroxymethylfurfural (HMF), 0.8 g/L furfural, 1.7 g/L acetic acid and 1 g/L formic acid. The total concentration of phenols was 3 g/L (calculated as vanillin equivalents).

NH_4OH Detoxification

A multilevel factorial experiment was designed for treatment of the spruce hydrolysate with NH_4OH at different pH values and temperatures using MODDE 7.0 software (Umetrics AB, Umeå, Sweden). Based on previous experience with NH_4OH *(8)*, $Ca(OH)_2$ *(6)* and NaOH treatments *(9,10)*, different pH-conditions (pH 8.0, 9.0, and 10.0) and different temperatures (5, 30, 42.5, 55, and 80°C) were investigated in different combinations during 3 h. Treatments were not performed over pH 10.0 owing to the pK_a of ammonium (9.25 at 25°C). The 13 different conditions are shown in Table 1. The treatments were performed using a TIM 900 Titration Manager (Radiometer Analytical, Copenhagen, Denmark) in pH-static mode. The pH-meter was corrected against the temperature before use, as the pH is influenced by the temperature and incorrect pH values otherwise will result at temperatures higher than 25°C. After the different treatments, the pH was adjusted to 5.5 by addition of H_2SO_4. Because all the samples had been treated in different ways, water was added to give a final dilution of 5% for all hydrolysate samples.

NaOH Detoxification

A reduced multilevel factorial experiment was designed for treatment of the spruce hydrolysate at different combinations of pH and temperature using the MODDE 7.0 software (Umetrics). The different combinations of pH (9.0, 10.0, 11.0, and 12.0) and temperature (30, 55, and 80°C) used for treatment during 3 h are shown in Table 2. The treatments were performed in pH-static mode as described above. After the different treatments, the

Table 1
Concentrations of Inhibitors and Sugars After NH_4OH Detoxification and Assessment of Fermentability of the Treated Hydrolysates

Treatment conditions Temperature (°C)/pH		Inhibitors and sugars (% of conc. in untreated sample)					Assessment of fermentability Ψ_{EtOH} (% of reference fermentation)
		Furan aldehydes			Sugars		
		HMF	Furfural	Phenols	Glucose	Mannose	
5	8	95	92	91	93	94	33
5	8	97	96	94	97	99	35
30	8	89	91	83	96	98	54
55	8	83	73	93	93	94	109
80	8	69	49	128	95	92	97
5	9	75	81	104	94	94	60
30	9	77	66	90	99	102	77
42.5	9	67	68	100	93	95	103
42.5	9	63	65	102	90	91	109
55	9	68	66	87	93	94	120
80	9	57	44	109	88	90	111
5	10	78	80	82	95	96	42
30	10	68	69	95	94	96	44
55	10	50	43	88	92	93	102
80	10	11	7	126	75	84	99
80	10	4	2	112	73	86	102
Untreated		100	100	100	100	100	7

pH was adjusted to 5.5 by addition of HCl and the volume was adjusted with water so that the total dilution was always 5%.

Combined NaOH/NH_4OH Detoxification

To investigate the role of ammonium and the importance of pH adjustment during detoxification, a combined NaOH/NH_4OH treatment was performed. Different doses of NH_4OH (2.5, 15, 30, 45, and 55 mmol) were added to 114 mL of the hydrolysate. Then, NaOH was added until the pH reached 10.15. The sample to which 55 mmol of NH_4OH was added reached pH 10.15 directly and in that case no addition of NaOH was made. In addition, one hydrolysate sample was treated with only NaOH at 10.15. All samples were treated at 55°C for 3 h. After the treatment, the pH was adjusted to 5.5 by addition of HCl. The volume of all samples was adjusted with water so that the final dilution was 5%.

Simultaneous Comparison of Detoxification With Different Types of Alkali

As the optimization experiments with NH_4OH and NaOH (this study) and $Ca(OH)_2$ *(6)* were conducted at separate occasions and using separate

Table 2
Concentrations of Inhibitors and Sugars After NaOH Detoxification and Assessment of Fermentability of the Treated Hydrolysates

Treatment conditions		Inhibitors and sugars (% of concectration in untreated sample)							Assessment of fermentability
		Furan aldehydes			Aliphatic acids		Sugars		Ψ_{EtOH} (% of reference
Temp.(°C)	pH	HMF	Furfural	Phenols	Acetic	Formic	Glucose	Mannose	fermentation)
55	9	78	99	96	90	82	94	93	19
55	9	79	102	96	96	85	96	93	27
80	9	71	63	88	98	93	94	89	111
30	10	63	93	97	97	93	95	92	18
55	10	52	49	105	105	104	103	97	77
80	10	65	36	107	107	117	72	72	74
30	11	30	32	94	94	89	92	88	80
55	11	<1	<1	127	102	108	75	88	82
80	11	2	<1	156	115	130	51	61	74
30	12	5	5	114	101	103	84	95	65
30	12	5	6	111	99	101	89	100	80
55	12	0	0	168	133	166	35	34	63
Untreated		100	100	100	100	100	100	100	6

inocula, an experimental series was performed in which selected conditions for treatment with $Ca(OH)_2$, NH_4OH, and NaOH were applied and the detoxified samples were fermented in parallel. The samples were treated for 3 h using the pH-stat as previously described. The conditions were: NaOH, 80°C and pH 9.0; NH_4OH, 55°C and pH 9.0; $Ca(OH)_2$, 30°C and pH 11.0. The total amounts of added alkali to achieve and maintain the conditions described were approx 20 mmol of NaOH, 50 mmol of NH_4OH, and 15 mmol of $Ca(OH)_2$. The subsequent adjustment of the pH to 5.5 required an addition of HCl of 1.5 mmol for the NaOH-treated sample, 20 mmol for the NH_4OH-treated sample, and 10 mmol for the $Ca(OH)_2$-treated sample.

Yeast Strains and Growth Conditions

The fermentations were carried out using *Saccharomyces cerevisiae* (Jästbolaget AB, Rotebro, Sweden). Agar plates with yeast extract peptone dextrose (YEPD) medium (2% yeast extract, 1% peptone, 2% D-glucose, and 2% agar) were used to maintain the strain. Cultures for preparing inocula were grown in 2000 mL cotton-plugged Erlenmeyer flasks containing 1200 mL YEPD medium. The flasks were incubated with agitation at 30°C for approx 12 h. Cells were harvested in the exponential phase by centrifugation (Sorvall RC26 Plus, Dupont) at 1500*g* and 4°C for 5 min. Thereafter, the cells were washed with a sodium chloride solution (9 g/L) and centrifuged as before. To determine the dry weight of the inoculum, a membrane filter (0.45 µm HA filter, Millipore, Milford, MA) was dried in a microwave oven (Husqvarna Micronett, Sweden) set at a power scale of 3 for 15 min and thereafter placed in a desiccator. After 2 h, the filter was taken from the desiccator and weighed on an analytical scale. One and a half milliliters of the yeast suspension were then filtered through the dried filter under the influence of suction. The filter was washed with 5 mL water, dried as previously described, and weighed.

The hydrolysate samples were filtered before fermentation to remove precipitate and the pH was 5.5. The hydrolysate sample (47.5 mL) (or, alternatively, 47.5 mL of a synthetic sugar solution in water for reference fermentations) was mixed with 1 mL of a nutrient solution (consisting of 50 g/L yeast extract, 25 g/L $(NH_4)_2HPO_4$, 1.25 g/L $MgSO_4$ 7 H_2O, 79.4 g/L NaH_2PO_4 H_2O) and 1.5 mL of the inoculum (adjusted to give an initial biomass concentration of 2 g/L dry weight [DW]) in each fermentation vessel. The fermentation vessels were equipped with magnets for stirring and sealed with rubber stoppers with cannulas for outlet of CO_2. The vessels were then placed in an incubator at 30°C with magnetic stirring. The glucose levels during the fermentation were monitored by using a glucometer (Glucometer Elite XL, Bayer AG, Leverkusen, Germany). Samples (0.2 mL) taken from the vessels were diluted with water (1.8 mL) and filtered through an HPLC filter (0.45 µm GHP Acrodisc 13 mm syringe filter, Pall, Ann Arbor, MI) and stored at –20°C until analyzed.

Analysis of Hydrolysates

The concentrations of glucose and mannose were determined as previously described *(11)* using high-performance anion-exchange chromatography (HPAEC) with a DX 500 chromatography system (Dionex, Sunnyvale, CA) equipped with a CarboPac PA-1 column (Dionex).

HMF and furfural were analyzed using HPLC with a 2690 separation module from Waters (Milford, MA) equipped with a binary pump, an auto injector, and a photo diode 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]) using conditions previously described *(11)*.

Quantification of acetic acid and formic acid was done using ion chromatography (IC). Before analysis, all samples were filtered through a 0.45 µm Acrodisc syringe filter (Pall Gelman Laboratory, Ann Arbor, MI) and diluted with Milli-Q water. The analysis was done by HPAEC with a Dionex ICS-2000 chromatography system with a conductivity detector, using an IonPac AS 15 (4 × 250 mm) analytical column and an IonPac AG15 (4 × 50) guard column (all from Dionex). An isocratic eluent concentration of 35 m*M* sodium hydroxide and a flow rate of 1.2 mL/min was used for separation of the anions. External calibration curves were used for the quantification. The total run time of the analysis was 16 min.

The total concentration of phenols was determined using a spectrophotometric method *(12)*, which is based on Folin and Ciocalteu's reagent (Sigma, Steinheim, Germany). Vanillin was used as the standard.

Analysis of Fermentation Samples

The concentrations of glucose and mannose were determined by HPAEC as previously described. The concentration of ethanol was measured using an HP 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a BP-20 column (film thickness of 1 µm) (SGE, Austin, TX) and a flame ionization detector *(11)*.

Results

The conditions and results of the NH_4OH optimization experiments are shown in Table 1. The software MODDE 7.0 was used for the evaluation of the effect on sugars, inhibitors and precipitate formation. The degradation of fermentable sugars was generally moderate, as shown in the graph for mannose in Fig. 1A. In contrast, the concentrations of HMF and furfural decreased rapidly under conditions with high pH and temperature, as indicated in Fig. 1C,D. The total concentration of phenols decreased slightly except for the treatments performed at 80°C where it increased. The concentrations of acetic and formic acid were only measured for the samples 5°C/pH 8.0 and 80°C/pH 10.0. No significant difference between the two samples was detected. The balanced ethanol yield is graphically

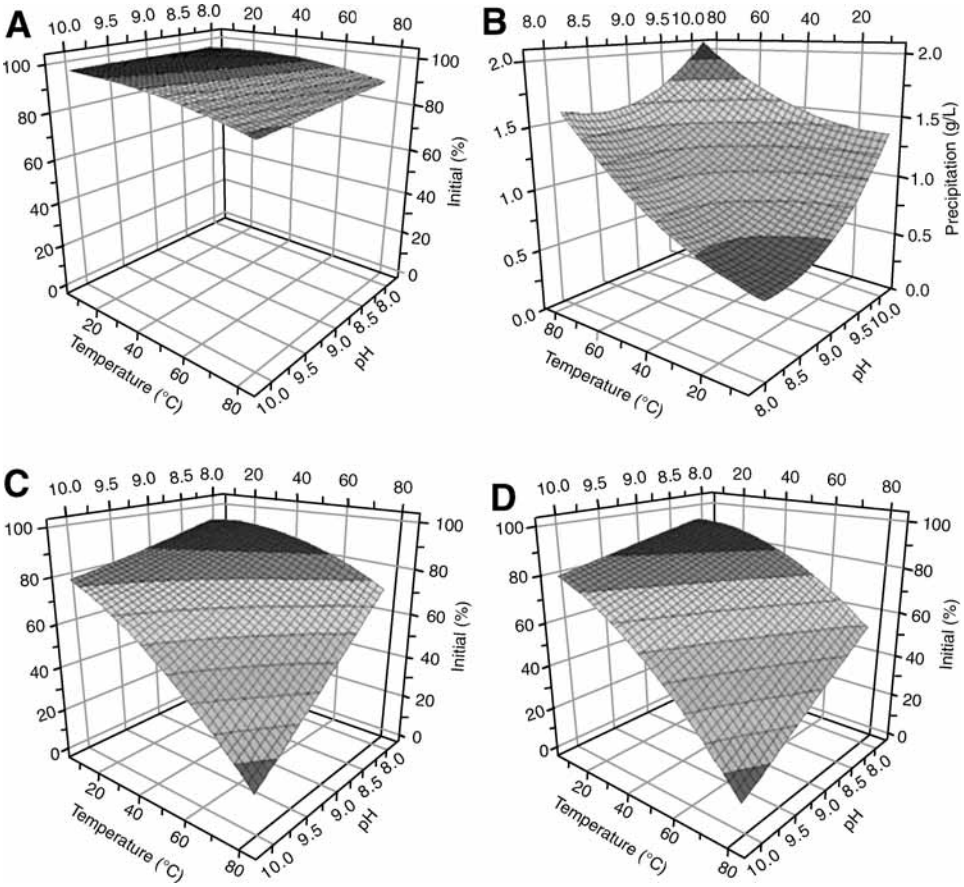

Fig. 1. Response surfaces of NH_4OH detoxification (generated by MODDE 7.0) for **(A)** mannose, **(B)** formation of precipitate, **(C)** HMF, and **(D)** furfural.

illustrated, using the software MatLab 7.0.4, in Fig. 2A. All treated samples displayed higher balanced ethanol yield than the untreated sample. The highest balanced ethanol yield was obtained after treatment at intermediate pH (approx 9.0) and intermediate-high temperature (approx 55°C) (Fig. 2A) and reached 120% of the value of the reference fermentation. During the treatments a brownish precipitate formed. The amount of precipitate increased with increasing pH and temperature (Fig. 1B).

The conditions and data used for finding optimal conditions for NaOH treatment are shown in Table 2. As expected *(10)*, the degradation of glucose and mannose was substantial under harsh conditions. Increase of temperature and pH within the intervals studied affected the degradation of the sugars similarly. The concentrations of HMF and furfural decreased under harsher conditions in a similar way. In the sample treated at 55°C/pH 12.0, no detectable levels of HMF or furfural were found. The total concentration of phenols decreased slightly under milder conditions but increased under harsh conditions. The concentrations of acetic and formic acid increased

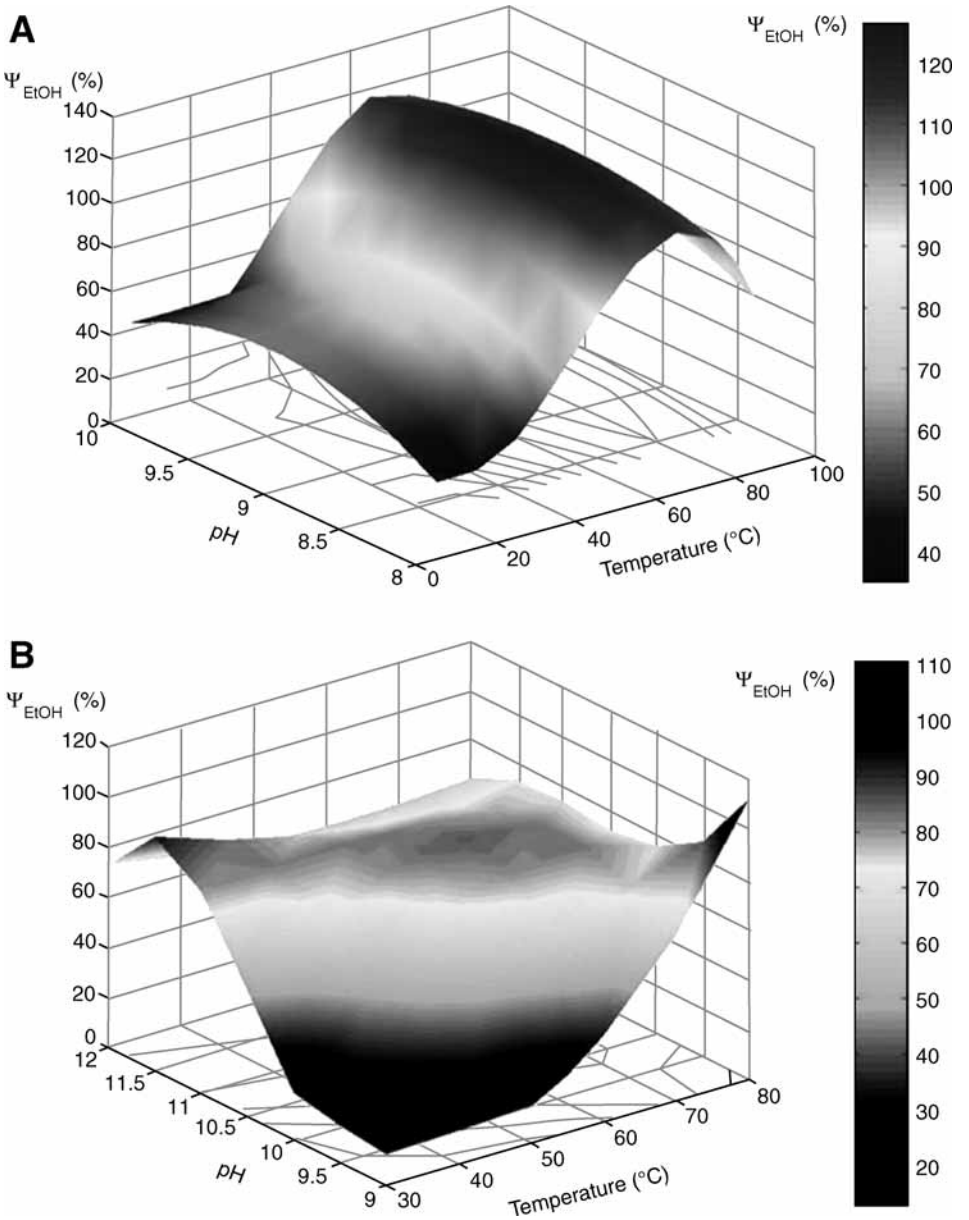

Fig. 2. The balanced ethanol yield (ΨEtOH) for **(A)** NH_4OH and **(B)** NaOH treatments, given in percent of the reference fermentation and plotted using MatLab 7.0.4.

under harsh conditions. The balanced ethanol yield is shown in Fig. 2B. All treatment conditions studied resulted in a higher balanced ethanol yield than for the untreated sample. The best conditions were high temperature in combination with moderate pH or moderate temperature in combination with high pH resulting in a ridge between these conditions, as displayed in Fig. 2B. The highest balanced ethanol yield corresponded to approx 110%

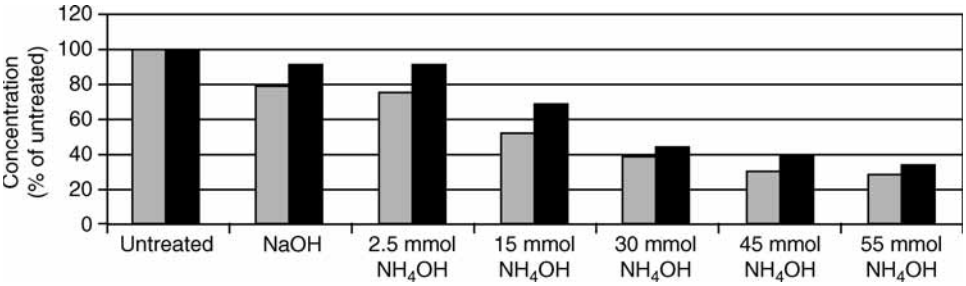

Fig. 3. The degradation of HMF (gray bars) and furfural (black bars) after treatment with NH_4OH, NaOH, or combinations thereof.

of the value of the reference fermentation. No significant amount of precipitate was formed during the NaOH treatments.

The combined experiment was carried out without the use of a pH-stat. This resulted in a decrease in pH for all samples during the 3 h treatment time. The largest drop in pH, from 10.15 to 7.8, was monitored in the sample that was treated only with NaOH. The smallest drop in pH, from 10.15 to 10.1, was monitored in the sample that was treated with 55 mmol of NH_4OH. The drop in pH for the rest of the samples increased with the decreasing amount of NH_4OH added. None of the samples to which NH_4OH was added dropped below pH 9.5. The concentrations of HMF and furfural decreased with the increasing amount of added NH_4OH (Fig. 3). The degradation ranged from 20% of the HMF and 8% of the furfural in the sample treated with only NaOH up to 70% of the HMF and 64% of the furfural in the sample treated with 55 mmol of NH_4OH. The degradation of phenols followed a similar pattern as that observed for furan aldehyde degradation, although it was less extensive, reaching a level of 12% in the sample treated with 55 mmol of NH_4OH. The concentrations of aliphatic acids were not measured. The fermentability was evaluated by comparing the glucose consumption for each sample during fermentation. For the samples with an NH_4OH addition of 15, 30, 45, and 55 mmol, all glucose was consumed within 4 h. For the sample with an NH_4OH addition of 2.5 mmol, all glucose was consumed within 6 h. In contrast, it took 28 h for all glucose to be consumed in the NaOH-treated sample.

Detoxification with $Ca(OH)_2$, NH_4OH, and NaOH was compared in a parallel fermentation experiment. The balanced ethanol yield for all detoxifications was approximately equal to or even better than that of the reference fermentation (Fig. 4). In contrast, the balanced ethanol yield of the untreated sample reached only 14% of that of the reference fermentation.

Discussion

Although overliming is an efficient detoxification method, there are several types of alkali that tentatively could be used as alternatives, especially

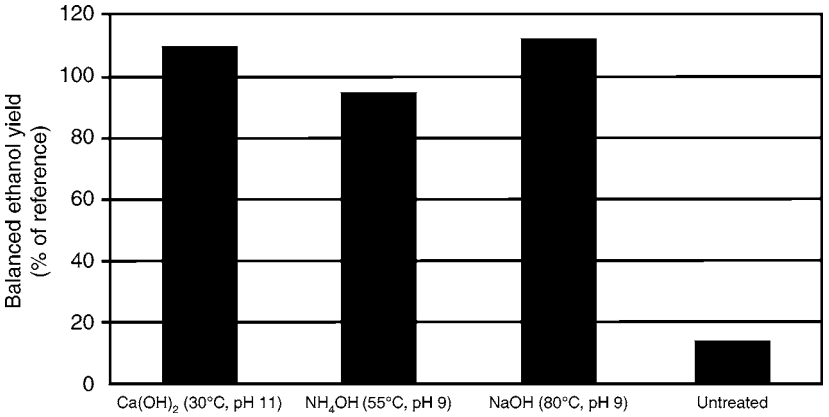

Fig. 4. Balanced ethanol yield in the experimental series in which hydrolysate samples detoxified with $Ca(OH)_2$, NH_4OH, and NaOH were fermented in parallel.

as overliming may be associated with problems that are not acceptable for industrial implementation, such as simultaneous degradation of fermentable sugars, resulting in poor ethanol yield, and formation of gypsum. As expected from previous results with NH_4OH *(4,8)*, it was possible to achieve excellent fermentability combined with low sugar degradation (Fig. 2A). The ethanol productivity and ethanol yield of some of the NH_4OH-treated samples even exceeded the values obtained for the reference fermentation, which has previously also been reported after optimization of overliming *(6)*. This can be explained by the presence of aliphatic and aromatic acids that in proper concentrations stimulate ethanol production at the expense of biomass formation *(13–16)*. Under the relatively mild conditions that were used for detoxification with NH_4OH, only small differences in the concentrations of aliphatic acids were observed, which can be attributed to the importance of sugars as precursors to aliphatic acids in the alkaline sugar degradation *(17)*. In these experiments only minor sugar degradation occurred (Fig. 1A). If the sugar degradation and the levels of aliphatic acid are compared at the same temperature and pH (this work and *[6]*), there are no large differences between NH_4OH, NaOH, and $Ca(OH)_2$ treatment. The chemical composition of the precipitate formed in the experiments with NH_4OH was not established. The amount of furan aldehydes decreased whereas the amount of precipitate increased, which suggests that a reaction between furan aldehydes and ammonia may have occurred. It has previously been suggested that the furan aldehydes may be involved in alkali-assisted aldol condensation reactions *(6)*. Advantages with NH_4OH include that the amount of precipitate formed using NH_4OH is lower than the amount of gypsum formed using $Ca(OH)_2$, relatively mild conditions can be used with NH_4OH, and the removal of furan aldehydes and phenols is relatively extensive.

Detoxification with NaOH did not give rise to any precipitate as in detoxification with $Ca(OH)_2$ and NH_4OH. No significant amount of precipitate

was observed regardless whether H_2SO_4 or HCl was used for the pH adjustments. The absence of precipitation might be owing to several factors, one being the low degree of removal of furan aldehydes. Precipitation of toxic factors has been suggested to be an explanation for good detoxification results achieved with calcium salts *(18)*. Other studies suggest that chemical conversions rather than coprecipitation are important for removal of inhibitors *(4)*. Our study shows that precipitation is not prerequisite for efficient detoxification.

The experimental series in which NH_4OH and NaOH was combined shows that one of the benefits of NH_4OH is the buffering effect which keeps the pH on a high and stable level during the detoxification. Even the addition of an amount of NH_4OH corresponding to only 21 m*M* (the 2.5 mmol experiment) resulted in a much more stable pH. This result can explain that NH_4OH generally performs well if comparisons between different forms of alkali are performed without using pH-stat. The experiment also illustrates the importance of pH adjustment during detoxification with NaOH. Despite the differences in HMF and furfural levels observed in the different samples (Fig. 3), the fermentabilty of the four samples with a NH_4OH addition between 15 and 55 mmol was equally good. This indicates that neither of the furan aldehydes are important as inhibitors in the particular hydrolysate studied, which is also evident from the modest initial concentrations. Spruce hydrolysates may contain considerably higher concentrations of furan aldehydes *(1,19)*. Although the total levels of phenols did not change very much, there might be separate phenols in the hydrolysate that are more toxic to the yeast than others and who are affected differently by different kinds of alkali. Further studies are needed to answer this hypothesis.

The simultaneous comparison of samples detoxified using $Ca(OH)_2$, NH_4OH, or NaOH (Fig. 4) verified the results from the three separate optimization experiments (NH_4OH and NaOH, this work; $Ca(OH)_2$, *[6]*) in the sense that either type of alkali can be used to achieve high balanced ethanol yield, as long as optimal or near-optimal conditions are applied.

In conclusion, the results show that excellent fermentability combined with low sugar degradation can be achieved regardless of whether $Ca(OH)_2$, NH_4OH, or NaOH is used for alkali detoxification. NH_4OH treatment can be performed under mild conditions, gives a good buffering effect, low sugar degradation and extensive removal of inhibitors, particularly of furan aldehydes. NH_4OH gives rise to a precipitate, which was not observed for samples treated with NaOH. The choice of detoxification method is also dependent of other factors, such as the cost of different forms of alkali and possibly also costs for heating to treatment temperature. To avoid costs for heating, it should be attractive to perform alkaline treatment directly after dilute-acid hydrolysis, before cooling. The optimization experiment shows that treatment at low temperature is a feasible option. Further studies are needed to elucidate the composition

of the precipitate generated by NH_4OH treatment, the role of separate phenolic compounds as fermentation inhibitors, and the interconversion between different phenolic compounds that takes place during alkali treatment. The results obtained are an important step toward the development of an efficient detoxification procedure that is suitable for industrial implementation.

Acknowledgments

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