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Ammonium Hydroxide Detoxification of Spruce Acid Hydrolysates

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Abstract

When dilute-acid hydrolysates from spruce are fermented to produce ethanol, detoxification is required to make the hydrolysates fermentable at reasonable rates. Treatment with alkali, usually by overliming, is one of the most efficient approaches. Several nutrients, such as ammonium and phosphate, are added to the hydrolysates prior to fermentation. We investigated the use of NH_4OH for simultaneous detoxification and addition of nitrogen source. Treatment with NH_4OH compared favorably with $Ca(OH)_2$, $Mg(OH)_2$, $Ba(OH)_2$, and NaOH to improve fermentability using *Saccharomyces cerevisiae*. Analysis of monosaccharides, furan aldehydes, phenols, and aliphatic acids was performed after the different treatments. The NH_4OH treatments, performed at pH 10.0, resulted in a substantial decrease in the concentrations of furfural and hydroxymethylfurfural. Under the conditions studied, NH_4OH treatments gave better results than $Ca(OH)_2$ treatments. The addition of an extra nitrogen source in the form of NH_4Cl at pH 5.5 did not result in any improvement in fermentability that was comparable to NH_4OH treatments at alkaline conditions. The addition of $CaCl_2$ or NH_4Cl at pH 5.5 after treatment with NH_4OH or $Ca(OH)_2$ resulted in poorer fermentability, and the negative effects were attributed to salt stress. The results strongly suggest that the highly positive effects of NH_4OH treatments are owing to chemical conversions rather than stimulation of the yeast cells by ammonium ions during the fermentation.

Index Entries: Ethanol; lignocellulose; detoxification; NH_4OH; nitrogen.

Introduction

Overliming, treatment with $Ca(OH)_2$, is one of the most effective and widely used methods for detoxification of lignocellulose hydrolysates prior

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to fermentation for the production of fuel ethanol. The treatment is typically performed by adding $Ca(OH)_2$ to a pH of about 9.0 or 10.0 *(1,2)*. Other forms of alkali, such as NaOH and NH_4OH, have also been employed. When the effects of some different forms of alkali—NaOH, KOH, $Ca(OH)_2$, and NH_4OH, were studied, the NH_4OH treatment appeared to be one of the most promising methods *(3)*. Overliming also performed well in that comparison, but a potential drawback with overliming is the generation of precipitated gypsum (calcium sulfate).

Ammonium salts are usually added to lignocellulose hydrolysates as a nitrogen source for the fermenting microorganism. It is not clear from previous studies whether the positive effects of NH_4OH treatment of hydrolysates are a consequence of reactions taking place at alkaline conditions or of the extra addition of a nitrogen source to the hydrolysate.

Degradation of fermentable sugar may be a drawback when hydrolysates are treated under too harsh alkaline conditions. The limits for treatment with NaOH with respect to the concentrations of sugars and inhibitors have been examined *(4)*, whereas less is known about other alkaline treatments.

In the present study, several experimental series were designed to address fundamental questions concerning the treatment of lignocellulose hydrolysates with NH_4OH. A screening of treatments with different forms of alkali was performed to judge the performance of treatment with NH_4OH. Treatments with NH_4OH and $Ca(OH)_2$ were compared more in detail, in an attempt to separate the effects of additions of specific cations (ammonium and calcium) from the effects of reactions occurring at alkaline conditions. Treatments at alkaline conditions were performed at room temperature and pH 10.0, i.e., above the pK_a for ammonium (9.25 at 25°C). Thus, a substantial part was present as ammonia. The potential positive effect of the addition of an extra nitrogen source in the form of ammonium was studied. Furthermore, the effect of additions of salt to a hydrolysate on the performance of the fermenting microorganism, the yeast *Saccharomyces cerevisiae*, was investigated.

Materials and Methods

Hydrolysate and Chemicals

A dilute-acid hydrolysate of chipped Norway spruce, *Picea abies*, was prepared in a two-step hydrolysis process using sulfuric acid, as previously described *(5)*. The pH of the untreated hydrolysate was 1.9. The hydrolysate contained 17.9 g/L of glucose, 14.4 g/L of mannose, 3.1 g/L of hydroxymethylfurfural (HMF), 0.6 g/L of furfural, 1.2 g/L of levulinic acid, 2.8 g/L of acetic acid, and 1.0 g/L of formic acid. The total concentration of phenols was 5.1 g/L (as determined using Folin and Ciocalteu's reagent and vanillin as the standard).

Analysis (pro analysis)-grade chemicals were used. Reference fermentations were made using glucose. All fermentations were supplemented with 2% (v/v) of a nutrient solution giving final concentrations of 1 g/L of yeast extract, 0.5 g/L of $(NH_4)_2HPO_4$, 0.025 g/L of $MgSO_4 \cdot 7H_2O$, and 1.38 g/L of $NaH_2PO_4 \cdot H_2O$.

Treatment of Hydrolysate Samples

Series A

Series A was designed as an initial screening of the effect of alkaline treatment, using different mono- and divalent cations, on fermentability. Hydrolysate samples were adjusted to pH 10.0 (~22°C) using $Ba(OH)_2$, $Ca(OH)_2$, $Mg(OH)_2$, NaOH, or NH_4OH. The samples were kept at alkaline conditions for 1 h at ~22°C with magnetic stirring. The pH was then adjusted to 5.5 using HCl (37%). In parallel, an untreated hydrolysate sample was prepared by raising the pH from 1.9 to 5.5 using 3 *M* NaOH.

Series B

Series B was designed to compare different combinations of treatments with calcium and ammonium hydroxides and chlorides. Treatments at alkaline conditions were performed at 22°C during 3 h with magnetic stirring. The samples were as follows:

1. B1: untreated hydrolysate (the pH was adjusted to 5.5 using 5 *M* NaOH, i.e., no treatment at alkaline conditions).
2. B2: NH_4Cl (the pH was adjusted to 5.5 using 5 *M* NaOH, after which NH_4Cl was added to 320 m*M*).
3. B3: $CaCl_2$ (the pH was adjusted to 5.5 using 5 *M* NaOH, after which $CaCl_2$ was added to 90 m*M*).
4. B4: $Ca(OH)_2$ (the pH was adjusted to 10.0 using $Ca[OH]_2$, and after incubation, the pH was adjusted to 5.5 using 37% HCl).
5. B5: $Ca(OH)_2/NH_4Cl$ (the pH was adjusted to 10.0 using $Ca[OH]_2$, and after incubation, the pH was adjusted to 5.5 using 37% HCl, after which NH_4Cl was added to a concentration of 320 m*M*).
6. B6: NH_4OH (the pH was adjusted to 10.0 using 28% NH_4OH, and after incubation, the pH was adjusted to 5.5 using 37% HCl).
7. B7: $NH_4OH/CaCl_2$ (the pH was adjusted to 10.0 using 28% NH_4OH, and after incubation, the pH was adjusted to 5.5 using 37% HCl, after which $CaCl_2$ was added to a concentration of 90 m*M*).

Hydrolysate samples were filtered through a filter paper directly after the 3-h incubation at alkaline pH. After adjustment of the pH to 5.5, all samples were filtered again. Distilled water was used to equalize volume differences.

Series C

Series C was designed to test the effects of different amounts of a nitrogen source in the form of ammonium on fermentability. The pH of the hydrolysate was adjusted to 5.5 with 5 *M* NaOH. Different amounts of NH_4Cl(s) were then added to the hydrolysate samples to the following final concentrations: C1, no extra addition of nitrogen; C2, 1.0 g/L (19 m*M*); C3, 2.1 g/L (39 m*M*); C4, 3.4 g/L (64 m*M*); C5, 4.8 g/L (89 m*M*); C6, 7.4 g/L (140 m*M*); C7, 10.1 g/L (190 m*M*); C8, 12.8 g/L (240 m*M*); C9, 15.5 g/L (290 m*M*); C10, 18.2 g/L (340 m*M*); C11, 20.8 g/L (390 m*M*).

Series D

Series D was designed to test the effects of salt additions and conductivity on fermentability. The pH of the hydrolysate samples was adjusted to 10.0 using either NH_4OH or $Ca(OH)_2$. Then, the samples were kept at the alkaline pH for 3 h at ~22°C, after which the pH was adjusted to 5.5 using 37% HCl. After the pH adjustment to 5.5, salts were added according to the following scheme: D1, untreated hydrolysate (the pH was adjusted to 5.5 using NaOH, no treatment at alkaline conditions); D2, NH_4OH; D3, NH_4OH and 100 m*M* Na_2SO_4; D4, NH_4OH and 200 m*M* Na_2SO_4; D5, NH_4OH and 300 m*M* NaCl; D6, NH_4OH and 600 m*M* NaCl; D7, NH_4OH and 100 m*M* $MgCl_2$; D8, $Ca(OH)_2$; D9, $Ca(OH)_2$ and 100 m*M* Na_2SO_4; D10, $Ca(OH)_2$ and 200 m*M* Na_2SO_4; D11, $Ca(OH)_2$ and 300 m*M* NaCl; D12, $Ca(OH)_2$ and 600 m*M* NaCl; D13, $Ca(OH)_2$ and 100 m*M* $MgCl_2$.

Yeast Strains and Growth Conditions

The yeast used for the fermentations was *S. cerevisiae* (Jästbolaget AB, Rotebro, Sweden). Agar plates with YEPD medium (2% yeast extract, 1% peptone, 2% D-glucose, 2% agar) were used to maintain the strain. The cultures used for inocula were grown in 2000-mL cotton-plugged Erlenmeyer flasks containing 1200 mL of YEPD medium. The flasks were incubated for approx 12 h at 30°C with agitation. Cells were harvested in the exponential phase by centrifugation (Sorvall RC26 Plus; Dupont) at 1500*g* for 5 min at 4°C. Thereafter, the cells were washed with an NaCl solution (9 g/L) and centrifuged again as before.

To measure the dry weight of the inoculum, a membrane filter (0.45-µm HA filter; Millipore) was dried in a microwave oven (Husqvarna Micronett, set at a power scale of 3) for 15 min and thereafter placed in an exsiccator. After 2 h, the filter was taken out of the exsiccator and weighed on an analytic scale. One and a half milliliters of the yeast suspension was then filtered through the dried filter under suction. The filter was washed with 5 mL of H_2O, dried as previously described, and weighed.

Fermentations

The different hydrolysate samples and reference solutions were transferred to glass vessels for fermentation. The samples were supplemented

with 1 mL of nutrient solution (described in Hydrolysate and Chemicals) and 1.5 mL of inoculum (to give an initial cell mass concentration of 2 g/L dry wt), and the final volume was 50 mL. The fermentation vessels were equipped with magnets for stirring and sealed with rubber stoppers pierced by cannulas for release of CO_2. The vessels were then placed in an incubator with magnetic stirring. The temperature was kept at 30°C. The glucose levels during the course of the fermentation were measured with a glucometer (Glucometer Elite XL; Bayer AG, Leverkusen, Germany). Samples for chemical analyses (0.2 mL) were withdrawn, diluted with 1.8 mL of H_2O, filtered through a high-performance liquid chromatography (HPLC) filter (GHP Acrodisc 13-mm syringe filter with a 0.45-µm GHP membrane; Gelman Laboratory, Pall, Ann Arbor, MI), and placed in a –20°C freezer.

Analysis of Hydrolysates

High-performance anion-exchange chromatography (HPAEC) was used to determine glucose and mannose. The system, consisting of a DX 500 unit equipped with a CarboPac PA-1 column (Dionex, Sunnyvale, CA), was operated as previously described *(6)*.

Analysis of aliphatic acids was performed using a Beckman P/ACE MDQ capillary electrophoresis instrument equipped with a 60 cm × 50 µm id fused silica capillary (Beckman Coulter, Fullerton, CA) as previously described *(6)*. All samples were filtered through a 0.45-µm cellulose acetate filter (Whatman, Maidstone, UK) prior to injections.

HMF and furfural were analyzed using an HPLC system consisting of a Waters 2690 separation module, a binary pump, an autoinjector, and a photodiode array detector set at 282 nm (Waters, Milford, MA). The separation was carried out using an ODS-AL column (50 × 3 mm, 120 Å, 5-µm particles) (Waters) operated under conditions reported previously *(6)*.

The total concentration of phenols was estimated using a spectrophotometric method *(7)* based on Folin and Ciocalteu's reagent (Sigma, Steinheim, Germany). Vanillin was used as the standard.

The conductivity of selected hydrolysate samples was measured using a conductivity meter (CDM 80 Conductivity Meter; Radiometer, Copenhagen).

Analysis After Fermentation

The concentrations of the different monosaccharides were determined by HPAEC as described in the previous section. When only the consumption of glucose was followed, the glucometer was used. The ethanol concentration was measured using an HP 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a BP-20 column (film thickness of 1.0 µm) (SGE, Austin, TX) and a flame ionization detector *(7)*.

Assessment of Fermentability

The volumetric ethanol productivity (Q_{EtOH}, g ethanol/[L·h]) for the hydrolysate-containing samples was determined at the time point when

Table 1
Concentrations of Fermentation Inhibitors After Treatment with Different Types of Alkali[a]

Sample	Formic acid	Acetic acid	Levulinic acid	HMF	Furfural	Phenols
$Ba(OH)_2$	110	113	114	86	86	92
$Ca(OH)_2$	136	117	109	82	88	83
$Mg(OH)_2$	132	109	106	51	61	90
NaOH	117	115	113	92	95	90
NH_4OH	141	115	112	63	66	87
Untreated	100	100	100	100	100	100

[a]The concentrations of the fermentation inhibitors are given as the percentage of the concentrations in untreated hydrolysate. The samples were fermented as in series A.

the maximum volumetric ethanol productivity for the reference fermentation was recorded. A novel dimension, balanced ethanol yield (ψ_{EtOH}) *(8)*, which takes into account both the fermentability and the sugar degradation during alkaline treatments, was calculated as the amount of ethanol produced divided by the amount of fermentable sugar present in the sample before detoxification.

Results

In an initial series of experiments (series A), the impact of different types of alkali was screened. Chemical analysis of the effect of the treatments on different fermentation inhibitors showed that the concentrations of aliphatic acids (formic, acetic, and levulinic acid) either increased or remained very close to the initial levels (Table 1). The largest increase in the concentration of formic acid, about 40%, was observed in the samples treated with NH_4OH and $Ca(OH)_2$. The concentrations of the furan aldehydes (HMF and furfural) decreased after all forms of alkali treatment (Table 1). The smallest changes were observed for the NaOH-treated sample, in which >90% of the furan aldehydes remained. The samples treated with $Ca(OH)_2$ and $Ba(OH)_2$ displayed an intermediate decrease in the concentrations of furan aldehydes; eighty to ninety percent remained. The samples treated with $Mg(OH)_2$ and NH_4OH contained the lowest concentrations of furan aldehydes, ranging from 47 to 75% of the initial concentrations. In most cases, the decrease in the concentration of furfural was close to the decrease in HMF. The decrease in the concentration of phenols was most evident for the samples treated with $Ca(OH)_2$ and NH_4OH, in which about 85% of the initial concentration was left.

Glucose consumption during fermentation of the samples in series A (Fig. 1) showed that the sample treated with NH_4OH performed better than the sample treated with $Ca(OH)_2$ which, in turn, performed considerably

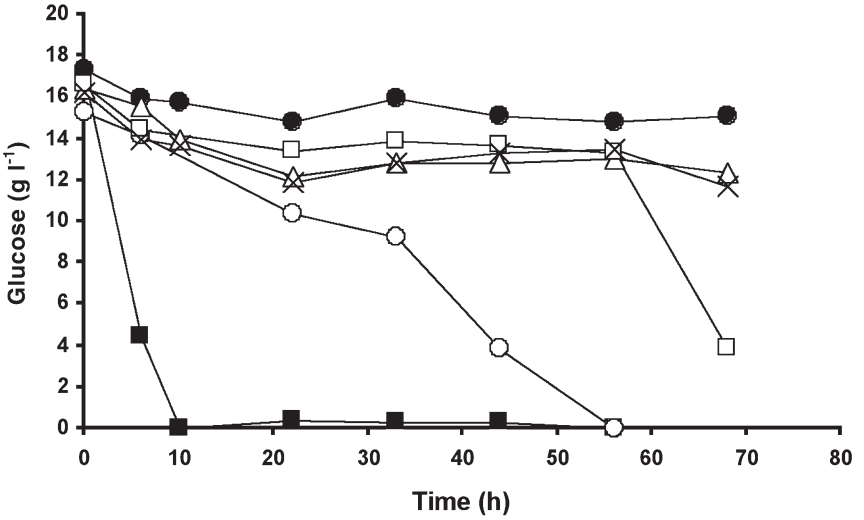

Fig. 1. Consumption of glucose in samples in series A: (■) NH_4OH; (❍) $Ca(OH)_2$; (□) $Ba(OH)_2$; (Δ) NaOH; (×) $Mg(OH)_2$; (●) untreated.

better than the samples treated with $Ba(OH)_2$, $Mg(OH)_2$, or NaOH. The $Ba(OH)_2$-treated sample started to ferment more rapidly at the end of the experiment. The differences among the samples were large. All glucose was consumed in the NH_4OH-treated sample after 10 h, whereas the NaOH- and $Mg(OH)_2$-treated samples showed a small decrease in glucose even after 70 h.

In a second series of experiments (series B), treatments with different combinations of hydroxides and chlorides of ammonium and calcium were compared; Figure 2 summarizes the results. The fermentability of the untreated hydrolysate (B1) was poor compared with a reference fermentation, in which a glucose solution was substituted for the hydrolysate. The volumetric ethanol productivity of the untreated hydrolysate reached only 7% of the productivity of the reference. The hydrolysate samples to which chloride salts were added showed no major improvement, compared with the untreated hydrolysate. The NH_4Cl- (B2) and $CaCl_2$-treated (B3) samples showed an ethanol productivity that was 9 and 8% of the productivity of the reference, respectively. All samples treated under alkaline conditions showed major improvements in fermentability. The $Ca(OH)_2$-treated (B4) sample displayed a productivity that was 41% of the reference value, and the addition of NH_4Cl after the $Ca(OH)_2$-treatment (B5) resulted in a lower ethanol productivity (31% of the reference value). The NH_4OH-treated sample (B6) again performed better than the $Ca(OH)_2$-treated sample (B4) and reached a productivity that was 86% of the reference value. The addition of $CaCl_2$ after NH_4OH treatment (B7) resulted in a poorer fermentability, and the sample reached a productivity that was 52% of the reference value.

The balanced ethanol yield (ψ_{EtOH}) at 12-h fermentation time for the samples in series B is shown in Fig. 2. The untreated sample (B1) and the

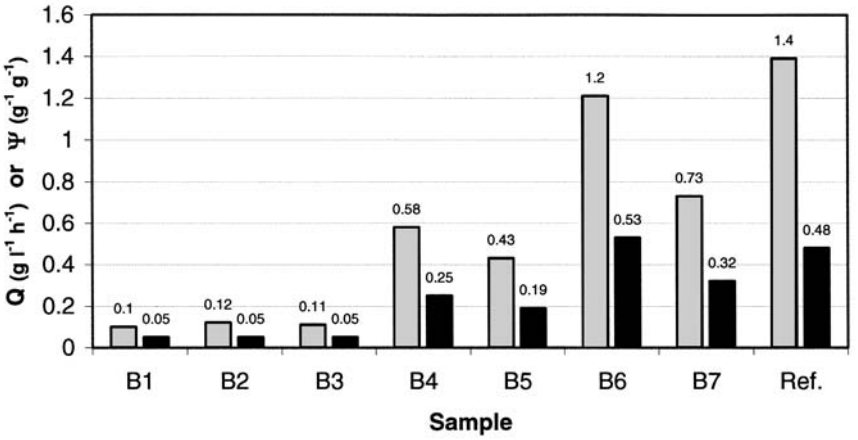

Fig. 2. Volumetric ethanol productivity (*Q*) (gray bars) and balanced ethanol yield (ψ) (black bars) for the samples: B1, untreated; B2, NH_4Cl; B3, $CaCl_2$; B4, $Ca(OH)_2$; B5, $Ca(OH)_2/NH_4Cl$; B6, NH_4OH; B7, $NH_4OH/CaCl_2$.

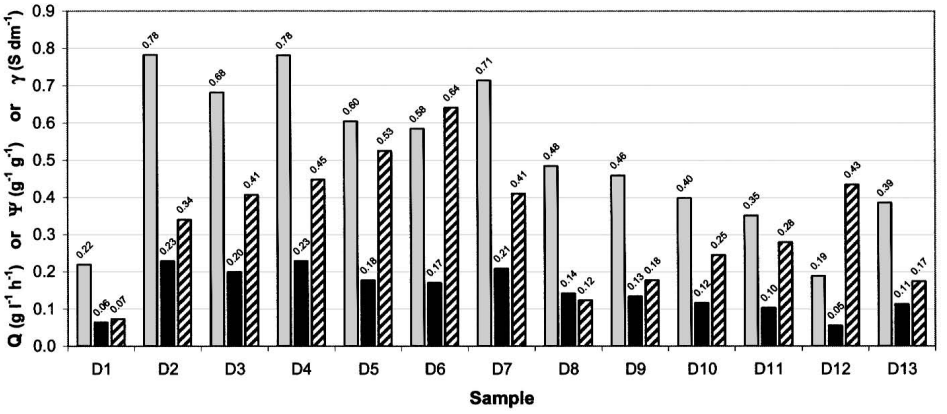

Fig. 3. Volumetric ethanol productivity (*Q*) (gray bars), balanced ethanol yield (ψ) (black bars), and conductivity (γ) (striped bars) for the samples: D1, untreated; D2, NH_4OH; D3, NH_4OH and 100 m*M* Na_2SO_4; D4, NH_4OH and 200 m*M* Na_2SO_4; D5, NH_4OH and 300 m*M* NaCl; D6, NH_4OH and 600 m*M* NaCl; D7, NH_4OH and 100 m*M* $MgCl_2$; D8, $Ca(OH)_2$; D9, $Ca(OH)_2$ and 100 m*M* Na_2SO_4; D10, $Ca(OH)_2$ and 200 m*M* Na_2SO_4; D11, $Ca(OH)_2$ and 300 m*M* NaCl; D12, $Ca(OH)_2$ and 600 m*M* NaCl; D13, $Ca(OH)_2$ and 100 m*M* $MgCl_2$.

two samples to which chlorides were added without treatment at alkaline conditions (B2 and B3) reached a balanced ethanol yield that was only 10% of the reference value. The $Ca(OH)_2$- (B4) and $Ca(OH)_2/NH_4Cl$-treated (B5) samples displayed a balanced ethanol yield that was 52 and 40% of the reference value. The values for the balanced ethanol yield (Fig. 2) are

based on data from the samples taken after 12 h, when the reference fermentation reached its highest value. At the end of the experiment, i.e., after 24 h of fermentation, the $Ca(OH)_2$-treated sample reached a balanced ethanol yield of 0.43 g/g (90% of the reference). The NH_4OH-treated (B6) sample showed a balanced ethanol yield that was even slightly better than the reference fermentation after 12 h (Fig. 2). The addition of $CaCl_2$ after NH_4OH treatment (B7) resulted in a balanced ethanol yield 67% of that of the reference (Fig. 2).

In series C, different amounts of NH_4Cl were added to the hydrolysate to determine whether a nitrogen source in the form of NH_4^+ could explain the powerful effect of the NH_4OH treatment. The extra addition of NH_4Cl, with final concentrations ranging between 1 and 20.8 g/L, did not result in any major improvement in fermentability (data not shown). The fermentations were followed during 24 h, and glucose was depleted in the reference fermentation after 10 h. In the hydrolysate-containing samples, >75% of the glucose still remained after 24 h, regardless of the concentration of NH_4Cl.

The samples in series B, which were treated at pH 10.0 and to which NH_4Cl or $CaCl_2$ was added after adjustment of the pH to 5.5, showed a poorer fermentability than the corresponding samples to which chlorides were not added (Fig. 2). A possible explanation could be salt stress, which was further explored in series D. Figure 3 shows the ethanol productivity, balanced ethanol yield, and conductivity after performing the detoxification treatment. The samples treated at alkaline pH using NH_4OH or $Ca(OH)_2$ (D2–D13) displayed a better fermentability than the untreated sample (D1), with the exception of sample D12 ($Ca[OH]_2$ + 600 m*M* NaCl). Ethanol productivity and balanced ethanol yield were always higher for the NH_4OH-treated samples (D2–D7) than for the corresponding $Ca(OH)_2$-treated samples (D8–D13). The fermentability of the samples containing extra addition of salts (D3–D7 and D9–D13) was equal to or poorer than the sample treated with the corresponding alkali (D2 and D8). The conductivity for the alkali-treated samples (D2–D13) was higher than for the untreated sample (D1). The conductivity for the samples treated with NH_4OH was always higher than that for the corresponding $Ca(OH)_2$-treated sample (Fig. 3).

Figure 4 shows fermentability, given as the ethanol productivity, against conductivity for the samples in series D. The untreated sample, which displayed low conductivity and poor fermentability, is situated apart from the others. The $Ca(OH)_2$-treated samples form a group with low to intermediate conductivity and poor to intermediate fermentability. There is a trend within this group that a lower conductivity corresponds to a better fermentability. The NH_4OH-treated samples form a group with intermediate to high conductivity and good fermentability. Within this group, there is a trend that lower conductivity is associated with better fermentability.

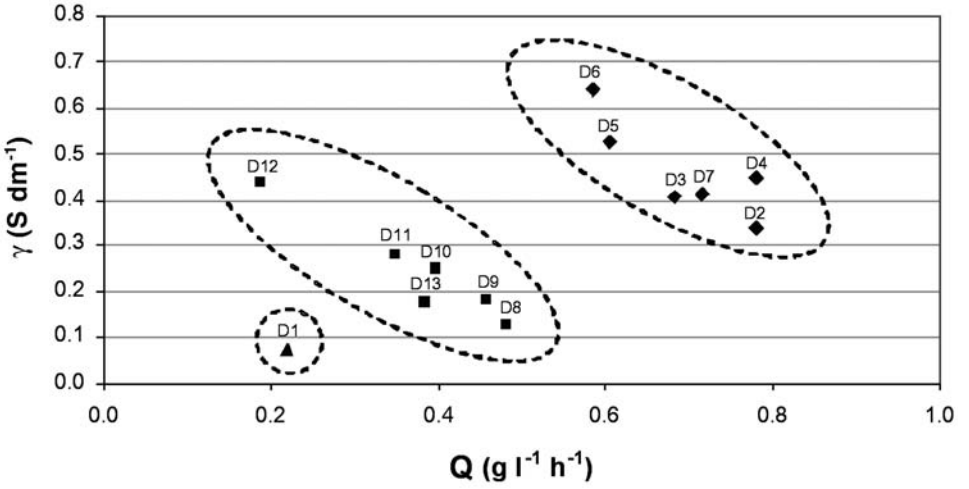

Fig. 4. Conductivity (γ) and ethanol productivity (*Q*) for samples in series D (see legend to Fig. 3): (▲) untreated sample; (■) $Ca(OH)_2$-treated sample; (◆) NH_4OH-treated sample.

Discussion

Treatments of dilute-acid hydrolysates of spruce, containing fermentation inhibitors, with NH_4OH at alkaline pH resulted in a dramatic improvement in fermentability. The results of the experimental series in which different types of alkali were compared (series A) suggested that detoxification with NH_4OH was worth studying in more detail, as an alternative to overliming. That series of experiments also indicated that hydroxides of divalent cations other than calcium, such as magnesium and barium, were ineffective. $Mg(OH)_2$ was also difficult to handle. The adjustment of pH to 5.5 after alkaline conditions was time-consuming owing to solubility problems, which also may have affected the result considering that the $Mg(OH)_2$-treated sample might have been exposed to a higher pH for a longer period of time than the other samples.

As expected, an increase in the concentration of aliphatic acids after treatment at alkaline conditions was observed. The generation of formic and acetic acid during treatment of dilute-acid hydrolysates of spruce with NaOH has previously been studied *(4)*. The concentrations of HMF and furfural decreased similarly (Table 1). The effect of alkali treatment on phenols is known to be very complex. The concentration of total and separate phenols may be unchanged, decrease, or increase, depending on the conditions, the form of alkali used, and the phenol studied *(3,4,9)*. Specific removal of phenols has proven their importance as inhibitors (e.g., see ref. 1), but more sophisticated methods are needed to characterize the phenol content of lignocellulose hydrolysates.

Van Zyl et al. *(10)* obtained better results in fermentation of sugarcane bagasse hydrolysates with *Pichia stipitis* when alkaline treatment at pH 10.0 was performed with $Ca(OH)_2$ instead of KOH. They compared NH_4OH with other types of alkali for adjustment of pH to 6.5, with discouraging results. However, treatment with NH_4OH under alkaline conditions was not studied *(10)*. Larsson et al. *(1)* compared detoxification with $Ca(OH)_2$ and NaOH. Under the conditions used, the treatment with $Ca(OH)_2$ performed better. Persson et al. *(3)* compared the fermentability of hydrolysate samples after treatment at pH 10.0 with NaOH, $Ca(OH)_2$, and NH_4OH. Fermentability was better after treatment with $Ca(OH)_2$ and NH_4OH than after treatment with NaOH. Taken together with the results from the present study, treatment of lignocellulose hydrolysates with $Ca(OH)_2$ or NH_4OH has, so far, showed better results than other forms of alkali. Which type of alkali treatment gives the best fermentability may possibly be owing to the conditions chosen for the comparison (pH, temperature, and time) or to the selection of feedstock or organism.

The possibility that the addition of NH_4^+ and Ca^{2+} ions, as such, supports the ability of the yeast to ferment toxic lignocellulose hydrolysates was explored in the experiments in series B. The results, however, indicated that the addition of NH_4Cl or $CaCl_2$ after the acidification following alkaline treatment resulted in poorer, rather than better, fermentability. This observation suggests that the positive effects of NH_4OH and $Ca(OH)_2$ treatments are to be sought under alkaline conditions. In agreement with this, the addition of NH_4Cl at pH 5.5 had no major effects.

The results from series B further suggested that conditions resulting in salt stress of the yeast may have been reached in the experiments in which chloride salts were added after alkaline treatments. The ionic composition of the hydrolysate is complex and the ionic strength is therefore difficult to estimate, but the conductivity was measured for the different samples in series D. Results from studies of bagasse hydrolysates *(2)* have shown decreased conductivity after overliming. This might be explained as precipitation effects, because calcium and sulfate form gypsum. The results from series D indicate that there is a link between high conductivity and poor fermentability, but only within either the NH_4OH or the $Ca(OH)_2$ series (Fig. 4). Presumably, the positive effects of NH_4OH treatment are more important than the negative effects of the increase in the salt concentration. The response of the yeast cells to high salt concentrations includes export of salt via ion pumps and increased production and accumulation of glycerol (reviewed in ref. *11*). *S. cerevisiae* belongs to yeasts that have their tolerance maxima in the range of 1 to 2 *M* NaCl, and glucose sustains growth up to 1.5 *M* NaCl (reviewed in ref. *12*). The composition of the hydrolysate is not fully known, but the data in Figs. 3 and 4 indicate that the addition of 300 m*M* NaCl was sufficient to give a clear negative effect on the fermentability.

Our results showed that NH_4OH can be used as an alternative to overliming for detoxification of lignocellulose hydrolysates. Advantages include that the formation of gypsum can be avoided and that NH_4OH treatment has a dual effect; that is, it represents simultaneous detoxification and addition of a nitrogen source, which is included in the medium anyway. However, the results suggested that the reason for the effect of NH_4OH treatment may be owing to chemical conversions, rather than to extra addition of nitrogen source for the yeast cells. A positive effect of the gypsum precipitation caused by overliming is a decrease in conductivity, but our results showed that the positive effects of NH_4OH treatment are more important for improving the fermentability, at least with our feedstock and organism.

Acknowledgments

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