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Detoxification of lignocellulosic hydrolysates using sodium borohydride

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HIGHLIGHTS

- Inhibitors in lignocellulosic hydrolysates prevent efficient bioconversion.
- A new method for detoxification of lignocellulosic hydrolysates is presented.
- Sodium borohydride treatment detoxifies hydrolysates by reduction of inhibitors.
- No extra process step required: can be performed as chemical in situ detoxification.
- Indicates difference between inhibition of microbes and of enzymes.

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ABSTRACT

Addition of sodium borohydride to a lignocellulose hydrolysate of Norway spruce affected the fermentability when cellulosic ethanol was produced using *Saccharomyces cerevisiae*. Treatment of the hydrolysate with borohydride improved the ethanol yield on consumed sugar from 0.09 to 0.31 g/g, the balanced ethanol yield from 0.02 to 0.30 g/g, and the ethanol productivity from 0.05 to 0.57 g/(L × h). Treatment of a sugarcane bagasse hydrolysate gave similar results, and the experiments indicate that sodium borohydride is suitable for chemical in situ detoxification. The model inhibitors coniferyl aldehyde, *p*-benzoquinone, 2,6-dimethoxybenzoquinone, and furfural were efficiently reduced by treatment with sodium borohydride, even under mild reaction conditions (20 °C and pH 6.0). While addition of sodium dithionite to pretreatment liquid from spruce improved enzymatic hydrolysis of cellulose, addition of sodium borohydride did not. This result indicates that the strong hydrophilicity resulting from sulfonation of inhibitors by dithionite treatment was particularly important for alleviating enzyme inhibition.

1. Introduction

Dwindling oil supplies combined with increased demand suggest the need for alternative feedstocks for production of fuels, chemicals, and materials such as plastics. Replacing oil and other fossil resources with sustainable and renewable lignocellulosic raw materials is therefore an exciting opportunity (Ragauskas et al., 2006; Lynd et al., 2008; Sims et al., 2010). Lignocellulosic raw materials such as wood residues and sugarcane bagasse are attractive as feedstock, since they are plentiful and relatively inexpensive.

Lignocellulose, which consists mainly of polymers such as lignin, cellulose and hemicellulose, is a recalcitrant material that offers a challenging problem when it comes to conversion to fermentable sugars. Thermochemical pretreatment, which involves high temperatures and use of acids, alkali or other chemicals, is usually required to make the raw material accessible to hydrolytic enzymes such as cellulases and hemicellulases. Severe conditions used during pretreatment usually lead to partial breakdown of lignin and hemicellulose-derived sugars, and result in the formation of unwanted by-products that in sufficiently high concentrations inhibit both fermenting microorganisms and cellulose-degrading enzymes. Fermentation inhibitors include many different compounds that can be categorized into a few groups, such as aromatic (mostly phenolic) compounds, furan aldehydes, and aliphatic acids (Larsson et al., 1999). Phenolic compounds can also inhibit enzymatic hydrolysis of cellulose (Ximenes et al., 2010). Lignocellulosic hydrolysates contain varying concentrations of inhibitory compounds depending on the composition of the raw material used in the process, and the severity and type of pretreatment used. There are several ways to counteract problems with fermentation inhibitors. The use of resistant fermenting microbes or chemical or biological treatments for detoxification of slurries and hydrolysates have been investigated.

Detoxification, which involves different types of treatments of the hydrolysates have been shown to dramatically improve the fermentability of strongly inhibitory lignocellulosic hydrolysates

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(Alriksson et al., 2006, 2011). The main objection that has been raised against detoxification is the need for an additional process step that would make the bioalcohol process more costly (Hamelinck et al., 2005). Alriksson et al. (2011) showed that this objection does not necessarily hold true, since it was discovered that treatment with reducing agents, including dithionite and hydrogen sulfite, can greatly improve the fermentability of lignocellulose hydrolysates when added directly to the fermentation vessel in the presence of the fermenting microorganism, *Saccharomyces cerevisiae*. Cavka et al. (2011) later showed that detoxification with sulfur oxyanions, such as sulfite and dithionite, results in sulfonation of fermentation inhibitors, a mechanism that also converts them to highly hydrophilic charged molecules. Treatment of lignocellulosic hydrolysates with reduced sulfur compounds also has positive effects on *Escherichia coli* (Nieves et al., 2011).

In this study we have investigated the effects of sodium borohydride on lignocellulosic hydrolysates and we have also used mass spectrometry (MS) to study the effects on selected model inhibitors. Furthermore, the effects were also compared to those of sodium dithionite and sodium hydrogen sulfite, which previously were shown to be potent agents of detoxification (Alriksson et al., 2011). As treatment of inhibiting compounds with sodium borohydride by necessity will generate other products than the sulfonated compounds that were identified after treatment with sulfite or dithionite, it is of mechanistic interest to compare the efficiency of sodium borohydride with that of the sulfur oxyanions. Furthermore, as is also the case with sulfite and dithionite, sodium borohydride is an industrial chemical that can be considered for large-scale processes (Rittmeyer and Wietelmann, 2002). In addition, there is a connection between sodium borohydride and dithionite, since sodium borohydride is used in the production of dithionite. Thus, both scientific and technical reasons motivate the study of the effects of sodium borohydride on lignocellulosic hydrolysates.

2. Methods

2.1. Pretreatment and hydrolysis of lignocellulosic raw materials

The hydrolysates used in this study were produced from sugarcane bagasse or from chipped wood of Norwegian spruce (*Picea abies*). The raw materials were first pretreated thermochemically and the resulting slurries were then converted by enzymatic hydrolysis. The liquid fractions obtained after removal of the lignin-rich solid residues remaining after pretreatment and enzymatic hydrolysis are referred to as hydrolysates. The hydrolysates thus contain sugars derived from both hemicellulose and cellulose.

The pretreatment of bagasse and spruce was performed by SEKAB E-Technology in the Swedish biorefinery demonstration plant (Örnsköldsvik, Sweden). The bagasse was pretreated in continuous mode in a 30-L reactor, which was filled approx. to 50% during operation. The pressure was 14 bar (188 °C), and the bagasse was impregnated with SO_2 (0.3 kg SO_2/h, which corresponds to around 0.6% SO_2/kg of sugarcane bagasse (DW, dry weight)). The residence time in the reactor was 10 min, and the resulting pH was 2.1. Unbarked spruce wood chips were treated in a continuous mode in the same reactor, but at a pressure of 18 bar (204 °C). There was an addition of 1.2–1.3 kg SO_2/h, which corresponds to 1% SO_2/kg of spruce wood chips (DW). The residence time in the reactor was 7–8 min, and the resulting pH was 1.4–1.5. After pretreatment, the spruce and bagasse slurries were cooled and stored at 4 °C until further use.

The pH of the bagasse slurry was adjusted to 5.3 with a 5 M solution of sodium hydroxide. The dry-matter content of the bagasse slurry was 18.3%. Six 2-L shake flasks were filled with 950 g of bagasse slurry. The pH of the spruce slurry was also adjusted to 5.3 with a 5 M solution of sodium hydroxide. Four 2-L shake flasks were each filled with 950 g of spruce slurry. The dry-matter content of the spruce slurry was 12.1%.

Commercially available preparations of cellulase and cellobiase were added to the slurries. The cellulase preparation, which was from *Trichoderma reesei* ATCC 26921, had a stated activity of 700 endoglucanase units (EGU)/g (Sigma–Aldrich, Steinheim, Germany) and the loading was 319 EGU/g of solids (DW). The cellobiase preparation, Novozyme 188, had a stated activity of 250 cellobiase units (CBU)/g (Sigma–Aldrich) and the loading was 23 CBU/g of solids (DW). After addition of enzymes, the slurries were incubated with shaking (Kuhner Lab-Therm LT-X, A. Kühner AG, Birsfelden, Switzerland) at 45 °C and 110 rpm for 72 h.

After hydrolysis, the slurries were centrifuged (Allegra X-22R, Beckman Coulter, Brea, CA, USA) at 4500*g* for 10 min at a temperature of 4 °C. The pH of the liquid fractions, the hydrolysates, was adjusted to pH 2.0 with a 12 M solution of HCl. The hydrolysates were stored at −80 °C until further use.

The monosaccharide content of the bagasse hydrolysate was: 85.3 g/L glucose, 18.8 g/L xylose, 3.4 g/L mannose, 1.4 g/L arabinose, and 0.7 g/L galactose. The bagasse hydrolysate contained 7.7 g/L acetic acid, 4.5 g/L furfural, and 0.7 g/L HMF. The monosaccharide content of the spruce hydrolysate was 84.4 g/L glucose, 13.7 g/L mannose, 8.0 g/L xylose, 2.0 g/L galactose, and 1.9 g/L arabinose. The spruce hydrolysate contained 4.3 g/L acetic acid, 2.0 g/L furfural, and 1.7 g/L HMF.

2.2. Treatment of hydrolysates

The treatment of the lignocellulosic hydrolysates was performed in a similar way as the treatments performed with sulfur-containing reducing agents in previous studies (Alriksson et al., 2011; Cavka et al., 2011). Prior to the treatments, the pH was adjusted to 6.0 with a 5 M solution of sodium hydroxide. The treatment of the hydrolysates was performed in 30 mL glass vessels equipped with magnetic stirrer bars and placed on a magnetic stirrer plate (IKA-Werke, Staufen, Germany) at room temperature (20 °C). Sodium borohydride (fine granular for synthesis, $\geqslant$98%, Sigma–Aldrich) was added as a powder directly to each of the vessels in different concentrations and allowed to react during 20 min. All treatments and experiments were performed in duplicates.

2.3. Concentration experiments

Experiments with different additions of sodium borohydride to lignocellulosic hydrolysates were performed in order to investigate if the sodium borohydride had any positive effect on the fermentablity of these lignocellulosic hydrolysates. Twenty-four and a half milliliter of hydrolysate were transferred to 30-mL glass vessels with magnetic stirring, two drops of anti-foam were added to counteract surface tension, and the sodium borohydride was then added directly to the vessels. The concentrations of sodium borohydride that were used were based on the total amount of HMF and furfural in each of the hydrolysates, and set to correspond to concentrations ranging from 0.1 to 1 furan aldehyde equivalents. For the experiment with bagasse hydrolysate the concentrations studied were 7, 15, 23, 31, 39, 47, and 55 mM. For the spruce hydrolysate, the concentrations were 4, 10, 16, 22, 28, 34, and 40 mM.

2.4. Effect on S. cerevisiae and time of addition

Experiments with glucose in 50 mM sodium citrate buffer, pH 6.0, were performed to investigate the effects, positive or negative,

of sodium borohydride on *S. cerevisiae* in the absence of fermentation inhibitors. The procedure and concentrations used in this experiment were the same as for the experiment with the spruce hydrolysate described above.

Experiments investigating effects of timing the sodium borohydride additions were also performed with spruce hydrolysate and 23 mM sodium borohydride. The relation of the additions of sodium borohydride to the time of the inoculum was: 20 min before, 10 min before, at the same time as, 10 min after, and 20 min after. The experiments were performed in 30-mL glass flasks to which 24.5 mL medium were added prior to the addition of sodium borohydride.

2.5. Comparison with other reducing agents

Another set of experiments were performed to compare the detoxification effects of sodium dithionite, sodium sulfite, and sodium borohydride. These experiments were performed in parallel and with spruce hydrolysate as medium. The concentration of reducing agent was 15 mM and the addition was made 20 min before the inoculum.

2.6. Fermentation

Fermentation experiments were performed for each of the treatments described above to evaluate the effects of the additions of the reducing agents. For comparison, untreated hydrolysate was included in all of the fermentation experiments. In the comparison experiment, a reference fermentation with glucose medium was included with a medium containing an amount of glucose corresponding to that in the hydrolysates. The fermentations were carried out using yeast (*S. cerevisiae* Ethanol Red, Fermentis Ltd., Marcq-en-Baroeul, France). The yeast inoculum was typically added as a freeze-dried preparation directly to the fermentation vessels and to a final concentration of 2 g/L (DW). The fermentations were carried out in 30-mL glass flasks equipped with magnets for stirring and sealed with rubber plugs pierced with cannulas for release of carbon dioxide. The hydrolysate samples (24.5 mL), or, alternatively, the sugar solution used for reference fermentations, were added to the fermentation flasks along with 0.5 mL of a nutrient solution (150 g/L yeast extract, 75 g/L $(NH_4)_2HPO_4$, 3.75 g/L $MgSO_4{\cdot}7H_2O$, 238.2 g/L $NaH_2PO_4{\cdot}H_2O$), and yeast inoculum. The flasks were incubated at 30 °C in a water bath with magnetic stirring (IKA-Werke). Samples for measurement of sugars and ethanol were withdrawn during the fermentation. The glucose levels during the fermentation were estimated by using a glucometer (Glucometer Elite XL, Bayer AG, Leverkusen, Germany).

2.7. Effects on enzymatic hydrolysis

Experiments with pretreatment liquid from the spruce slurry were used to investigate if sodium borohydride would result in any improvement of enzymatic hydrolysis when added to the pretreatment liquid. These experiments were performed with the same equipment and experimental set up as the fermentation experiments described above. In these experiments, 22.5 mL of pretreatment liquid were treated with 15 and 30 mM of sodium borohydride or sodium dithionite. The treatments were performed for 20 min with stirring. After the treatment, 2.5 g of Avicel (Fluka Biochemika, Buchs Switzerland) and 1% (w/w) of each of Novozyme 188 and Celluclast 1.5 L (Novozymes, Bagsvaerd, Denmark) were added to the pretreatment liquid. Two sets of reference hydrolysis reactions in citrate buffer (50 mM, pH 5.0) were performed: one with 30 mM of sodium borohydride and one without any addition of reducing agent.

2.8. Analysis of sugars, furans and organic acids

Analyses of monosaccharides, furan aldehydes (furfural and 2-hydroxymethylfurfural (HMF)), and organic acids were performed by using high-performance liquid chromatography (HPLC). A Shodex SH-1011 column (6 μm, 8 × 300 mm) (Showa Denko, Kawasaki, Japan) was used in a YoungLin YL9100 series system (YoungLin, Anyang, Korea) equipped with a YL9170 series refractive index (RI) detector for analysis of glucose, mannose, galactose, HMF, and furfural. Elution was performed with isocratic flow of a 0.01 M aqueous solution of H_2SO_4. The flow rate was 1.0 mL/min and the column temperature was set to 50 °C. For analysis of xylose and arabinose, a Shodex SP-0810 column (7 μm, 8 × 300 mm) was used

Table 1
Results of fermentation experiments with bagasse and spruce hydrolysates after detoxification with $NaBH_4$.[a]

$NaBH_4$ (at start) (mM)	Main hexose fraction (glucose and mannose) at start (g/L)	Main hexose fraction (glucose and mannose) after 70 h (g/L)	Consumed sugar (glucose and mannose) (g/L)	Ethanol yield[b]	Balanced ethanol yield[c]	Ethanol productivity[d]
Bagasse						
7	88.7	76.7	12.0	0.13	0.02	0.02
15	88.7	75.9	12.8	0.14	0.02	0.03
23	88.7	70.3	18.3	0.23	0.05	0.06
31	88.7	3.6	85.1	0.30	0.28	0.36
39	88.7	5.4	83.3	0.34	0.32	0.41
47	88.7	10.5	78.2	0.35	0.31	0.40
55	88.7	24.5	64.2	0.28	0.20	0.25
Untreated	88.7	75.0	13.7	0.12	0.02	0.02
Spruce						
4	98.1	68.6	29.5	0.29	0.09	0.17
10	98.1	69.2	28.9	0.15	0.04	0.08
16	98.1	4.0	94.1	0.28	0.27	0.52
22	98.1	3.6	94.5	0.31	0.30	0.56
28	98.1	1.5	96.6	0.28	0.28	0.52
34	98.1	1.9	96.2	0.31	0.30	0.57
40	98.1	2.0	96.1	0.27	0.27	0.50
Untreated	98.1	71.3	26.8	0.09	0.02	0.05

[a] The table shows the values at start and after 70 h (bagasse hydrolysate) or 52 h (spruce hydrolysate) of fermentation. The relative standard deviation of the method that was used for monosaccharide analysis was estimated to <10%.
[b] g EtOH/g consumed glucose and mannose.
[c] g EtOH/g glucose and mannose prior to detoxification.
[d] (g EtOH × L^{-1} × h^{-1}).

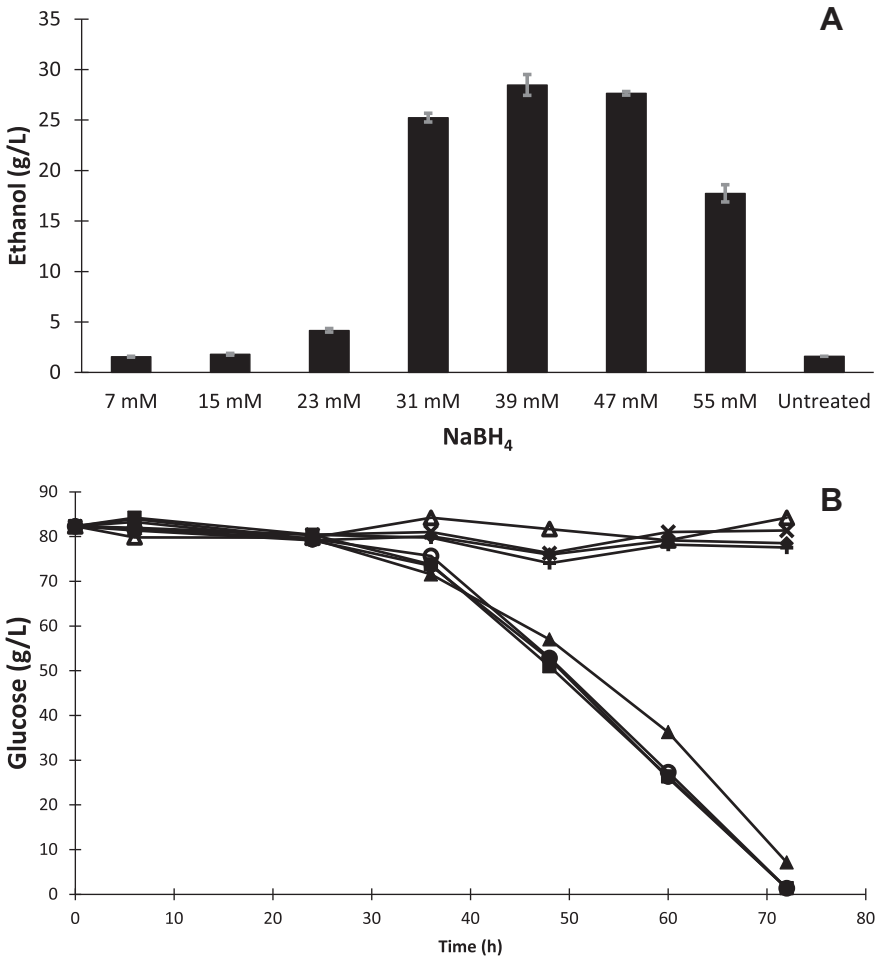

Fig. 1. Fermentation of a sugarcane bagasse hydrolysate in a separate hydrolysis and fermentation experiment with dried yeast as inoculum. (A) Ethanol production after 70 h. The bars show mean values of two fermentations, and the error bars indicate standard deviations. (B) Glucose consumption. Every point in the graph was calculated as the mean value of two separate fermentations. Sodium borohydride additions: untreated hydrolysate (Δ), 7 mM (♦), 15 mM (×), 23 mM (+), 31 mM (○), 39 mM (■), 47 mM (●), and 55 mM (▲).

with the same HPLC system. The elution was performed using Milli-Q water at a flow rate of 1.0 mL/min and the column temperature was set to 80 °C. YLClarity software (YoungLin, Anyang, Korea) was used for data analysis.

Ethanol measurements were performed by using an enzymatic kit (Ethanol UV-method, Boehringer Mannheim GmbH, Mannheim, Germany).

2.9. Treatment of model inhibitors

Aromatic model compounds (coniferyl aldehyde, ferulic acid, *p*-benzoquinone, 2,6-dimethoxybenzoquinone) and a furan aldehyde (furfural) were purchased from Sigma–Aldrich. Solutions of the model fermentation inhibitors were prepared at a concentration of 5 mM in citrate buffer (0.05 M, pH 6.0). The solutions were then treated with sodium borohydride (5 mM). Duplicates of treated and untreated samples were incubated for 20 min at room temperature (20 °C). All samples were stored at −80 °C until further analysis.

2.10. UHPLC-ESI–TOF-MS analysis

The analysis of the aromatic compounds and the furfural, before and after treatment with sodium borohydride, was performed using a Waters Acquity ultra high performance liquid chromatography (UHPLC) system, equipped with a column oven (held at 40 °C), and coupled to an LCT Premier time-of-flight mass spectrometer (TOF-MS) (Waters, Milford, MA, USA). Prior to analysis, duplicate samples were diluted 30:1 with deionized water to an approximate concentration of 15 μg mL^{-1}. Portions of 2 μL of each diluted sample were injected onto a 2.1 × 100 mm 1.7 μm C18 UPLC column (Waters). Elution was performed with a mixture of solutions A, which consisted of 0.1% formic acid in water, and B, which consisted of 0.1% formic acid in acetonitrile. The compounds were eluted at a flow-rate of 500 μL min^{-1} by using a mobile phase composed of 5% B over 3 min, 5–10% B (3–3.5 min), 10–30% B (3.5–4.5 min), 30–50% B (4.5–6.5 min), 50% B over 2 min, 50–95% B (8.5–9 min), and finally 95% B for 3.5 min. The mobile phase was then changed to 5% B after 12.5 min and the column was equilibrated for 3 min prior to the following sample injection. The eluate passed into the PDA (Photo Diode Array) detector (UV scanning from 210 to 500 nm at a sampling rate of 20 points s^{-1} and a resolution of 1.2 nm) and was then directly passed into the electrospray ionization (ESI) source. The source temperature was 120 °C, the cone gas flow was 10 L h^{-1}, the desolvation temperature was 320 °C, and the nebulization gas flow was 600 L h^{-1}. The capillary and cone voltages were set to 2.5 kV (negative ionization mode) and 35 V, respectively. Data were acquired in dynamic range enhancement (DRE) mode every 0.1 s and with a 0.01 s interscan delay. Leucine enkephalin was the lock mass compound for accurate mass measurements, and was infused directly at 500 pg μL^{-1} (in a 50:50 acetonitrile:H_2O mixture) and 30 μL min^{-1}. Mass spectra were acquired in centroid mode, *m*/*z* 100–1000, with a data threshold value set to 2.

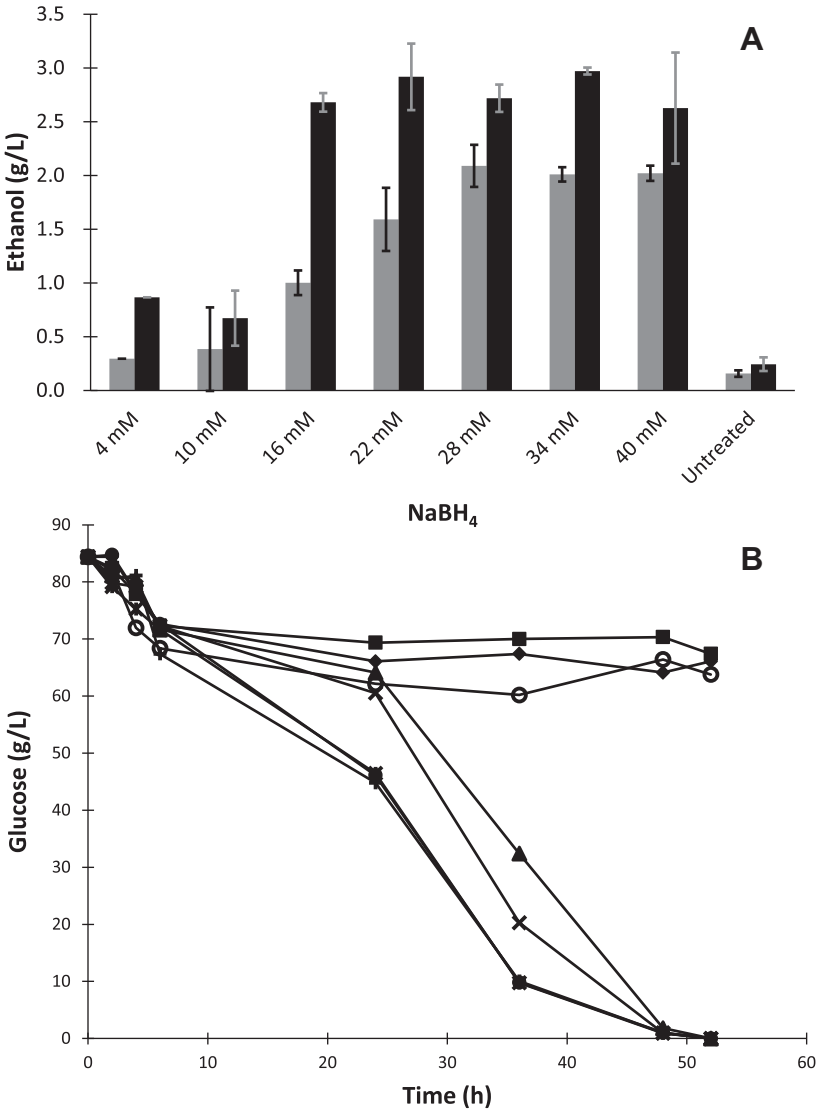

Fig. 2. Fermentation of a spruce hydrolysate in a separate hydrolysis and fermentation experiment with dried yeast as inoculum. (A) Ethanol production after 36 h (grey bars) and 52 h (black bars). The bars show the mean values of two fermentations, and the error bars indicate standard deviations. (B) Glucose consumption. Every point in the graph was calculated as the mean value of two separate fermentations. Sodium borohydride additions: untreated hydrolysate (○), 4 mM (♦), 10 mM (Δ), 16 mM (▲), 22 mM (×), 28 mM (■), 34 mM (●), and 40 mM (+).

Table 2
Comparison of detoxification effects of $NaBH_4$ and sulfur oxyanions.[a]

	Main hexose fraction (glucose and mannose) at start (g/L)	Main hexose fraction (glucose and mannose) after 21 h (g/L)	Consumed hexoses (glucose and mannose) (g/L)	Ethanol yield[b]	Balanced ethanol yield[c]	Ethanol productivity[d]
Sugar reference	100	<0.01	99.8	0.44	0.44	2.11
Sulfite	98.1	50.1	48.0	0.39	0.19	0.90
Dithionite	98.1	31.8	66.3	0.42	0.28	1.31
$NaBH_4$	98.1	30.9	67.2	0.42	0.29	1.35
Untreated	98.1	73.9	24.2	0.39	0.10	0.45

[a] The table shows the values at start and after 21 h of fermentation with conditioned yeast and spruce hydrolysate. The relative standard deviation of the method that was used for monosaccharide analysis was estimated to <10%.
[b] g EtOH/g consumed glucose and mannose.
[c] g EtOH/g glucose and mannose prior to detoxification.
[d] (g EtOH × L^{-1} × h^{-1}).

2.11. Data handling

Data acquired with the UHPLC-ESI–TOF-MS system were analyzed using the MassLynx software (Waters). When possible the compounds were quantified using the calculated peak areas of mass chromatograms, or otherwise a UV chromatogram was used. For quantification, mass windows of 0.1 Da of deprotonated negative ions (or, when present after ionization, deprotonated ions with formic acid adducts or dimer ions) were used. The quantification of aromatic compounds before and after treatment with sulfur oxyanions was based on the quantification of the same ions in both

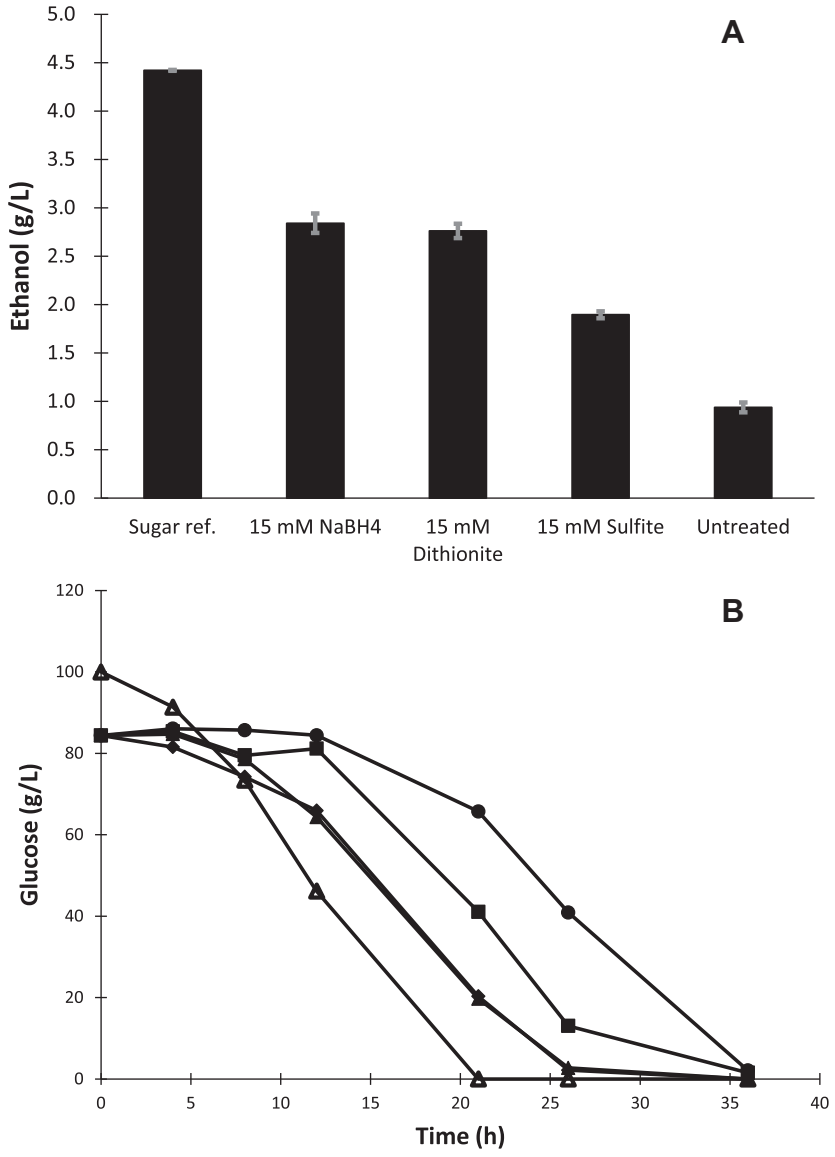

Fig. 3. Experiment with spruce hydrolysate in a separate hydrolysis and fermentation using conditioned yeast as inoculum. (A) Ethanol production after 21 h. The bars show mean values of two fermentations, and the error bars indicate standard deviations. (B) Glucose consumption. Every point in the graph was calculated as the mean value of two fermentations. The data points indicate: untreated hydrolysate (●), sodium sulfite treatment (15 mM) (■), sodium dithionite treatment (15 mM) (♦), sodium borohydride treatment (15 mM) (▲), and reference fermentation (Δ).

samples. This was done in order to avoid inaccurate quantification, which may arise from differences in response for the negative ions of the untreated compound compared to those of the resulting ions which arise after treatment. *p*-Benzoquinone, 2,6-dimethoxybenzoquinone, and furfural samples were quantified using UV data from the PDA detector rather than TOF-MS due to low ionization levels in ESI.

2.12. HPLC–UV–DAD

Detection and identification of products resulting from treatment of model fermentation inhibitors with sodium borohydride were performed using an HPLC–UV–DAD system set to scan between 210 and 500 nm (Agilent 1200 series, Agilent Technologies, Santa Clara, CA, USA). The system was equipped with a Nova-Pak C18 column (4 μm, 4.6 mm × 150 mm) (Waters Corporation, Milford, MA, USA). Isocratic elution with a mixture of 95% Milli-Q water and 5% acetonitrile (Sigma–Aldrich) was performed using a flow rate of 0.8 mL/min and an injection volume of 5 μL. Identification of resulting products after treatment with sodium borohydride was performed using both retention time comparisons with standards as well as database comparisons of the UV–DAD spectra with those available in the NIST database (The National Institute of Standards and Technology, Gaithersburg, MD, USA).

3. Results and discussion

3.1. Effects on fermentability

The effects of sodium borohydride were studied using lignocellulose hydrolysates prepared from sugarcane bagasse and Norway spruce. The sugars in the hydrolysates were derived from both hemicellulose, which was hydrolyzed during pretreatment, and from cellulose, which was released in the subsequent enzymatic hydrolysis. The glucose yields after 72 h of hydrolysis were similar, 85 g/L for the bagasse hydrolysate and 84 g/L for the spruce hydro-

lysate. The mannose yields were rather different (Table 1), which is expected considering that spruce wood has high mannan content.

The treatment of the lignocellulosic hydrolysates with sodium borohydride resulted in improved fermentability compared to untreated hydrolysates (Fig. 1). When the concentration of borohydride was raised to 31 mM, there was a sharp improvement in the fermentability of the bagasse hydrolysate (Fig. 1A). For the bagasse hydrolysate, 31 mM borohydride corresponded to 0.56 equivalents of furan aldehydes. As indicated by ethanol production (Fig. 1A) and glucose consumption (Fig. 1B), higher borohydride concentrations than 31 mM also resulted in a drastic improvement of the fermentability compared to the untreated reference, although 55 mM gave slightly less improvement than the additions in the range of 31–47 mM. Calculations of sugar consumption, ethanol yield on consumed sugar, balanced ethanol yield and ethanol productivity (Table 1) support that additions in the range of 31–47 mM gave the best results. After 70 h of fermentation, treatment of bagasse hydrolysate with sodium borohydride resulted in an increase in sugar consumption from about 14 to 85 g/L, an increase in ethanol yield on consumed sugar from 0.12 to 0.35 g/g, an increase in balanced ethanol yield from 0.02 to 0.32 g/g, and an increase in ethanol productivity from 0.02 to 0.41 g/(L × h). The fact that the treatment with 55 mM gave less improvement than treatments in the range of 31–47 mM indicates that an addition of sodium borohydride to 55 mM was slightly inhibitory for the fermenting microorganism.

The results of the experiments with spruce hydrolysate (Fig. 2, Table 1) were rather similar to those of the experiment with bagasse hydrolysate. The highest volumetric ethanol yields were achieved with an addition of 16 mM borohydride (which corresponded to 0.4 furan aldehyde equivalents, see Section 2.3) or higher concentrations, up to 40 mM (Fig. 2A). After 52 h fermentation, treatment of the spruce hydrolysate with sodium borohydride resulted in an increase in sugar consumption from about 27 to 97 g/L, an increase in ethanol yield on consumed sugar from 0.09 to 0.31 g/g, an increase in balanced ethanol yield from 0.02 to 0.30 g/g, and an increase in ethanol productivity from 0.05 to 0.57 g/(L × h) (Table 1).

The timing of the sodium borohydride additions and the subsequent effects on yeast were investigated in a set of experiments with spruce hydrolysate. These experiments were conducted to investigate the effects of a strong reducing agent, such as borohydride, on the fermenting microorganism, if it is present in the hydrolysate at the time of addition. The borohydride, which was added 20 and 10 min before, simultaneously, and 20 and 10 min after the addition of yeast to the spruce hydrolysate, showed no negative effects on the yeast's ability to consume glucose and produce ethanol. Experiments with citrate buffer solutions containing glucose in the absence of fermentation inhibitors were performed in order to investigate the toxicity levels of sodium borohydride on *S. cerevisiae*. The results showed that concentrations as high as 40 mM of sodium borohydride could be added to the buffer solution without any clear negative effects on either glucose consumption or ethanol productivity. This indicates that *S. cerevisiae* has high tolerance towards sodium borohydride, even in the absence of fermentation inhibitors.

Alriksson et al. (2011) showed that sulfur oxyanions could significantly improve the fermentability of lignocellulosic hydrolysates. Additions of as little as 5 mM of sodium dithionite resulted in improved fermentability compared to untreated control fermentations. The experiments with sodium borohydride (Figs. 1 and 2, Table 1) suggest that higher concentrations of the reducing agent are needed to achieve a maximal improvement of the fermentability, although the results are difficult to compare directly as the lignocellulosic hydrolysates used in the investigations differ. To obtain a comparison with the same hydrolysate, an experimental series including sodium borohydride, sodium sulfite and sodium dithionite was conducted using the spruce hydrolysate and 15 mM of the reducing agents. A reference fermentation containing similar amounts of fermentable sugars as in the spruce hydrolysate was included in the experiment. The results (Fig. 3, Table 2) showed that sodium borohydride treatment gave similar sugar consumption, ethanol yield on consumed sugar, balanced ethanol yield, and ethanol productivity as the treatment with dithionite (Table 2). The improvements achieved with sulfite were lower than the ones achieved with sodium borohydride and dithionite (Table 2). Data for volumetric ethanol yield and glucose consumption (Fig. 3) support that the effects of treatments with borohydride and dithionite were similar, while the effects of the treatment with sulfite were lower. These experiments were performed with conditioned yeast (the dried yeast was suspended in YPD medium and was kept for 3 h at 30 °C before inoculation) rather than with dried, which was used in all other experiments, and this can explain differences between different experimental series. The lag phase which was observed in experiments performed with dried yeast was not evident in experiments conducted with conditioned yeast.

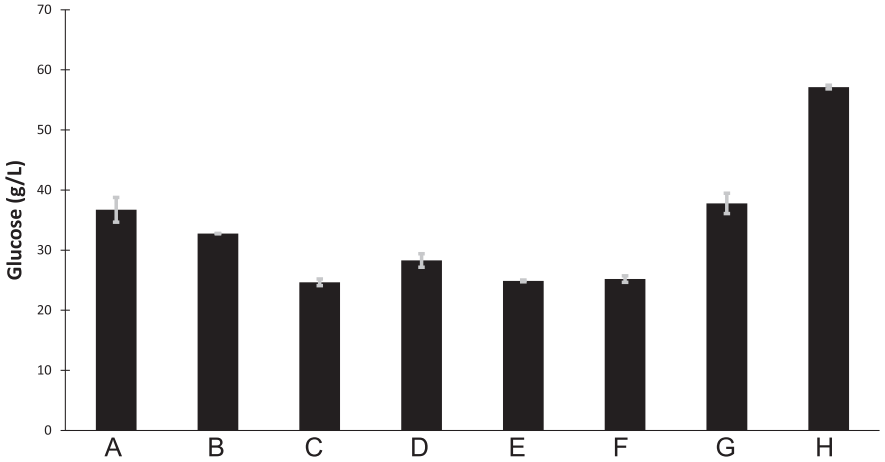

Fig. 4. Glucose yields after 72 h of enzymatic hydrolysis of 5% Avicel as substrate in pretreatment liquid from spruce or bagasse slurries. The bars show mean values of two separate hydrolysis experiments, and the error bars indicate standard deviations. (A) Spruce hydrolysate, 15 mM sodium dithionite; (B) spruce hydrolysate, 30 mM sodium dithionite; (C) spruce hydrolysate, 30 mM sodium borohydride; (D) spruce hydrolysate, untreated; (E) bagasse hydrolysate, 30 mM sodium borohydride; (F) bagasse hydrolysate, untreated; (G) buffer, 30 mM sodium borohydride and (H) buffer, untreated.

3.2. Effects on enzymatic hydrolysis

The effect of sodium borohydride on enzymatic hydrolysis was studied in another set of experiments. Previous studies show that reducing agents, such as the sulfur oxyanions dithionite and sulfite, and the sulfhydryl reagent DTT (dithiothreitol) improve enzymatic hydrolysis in the presence of pretreatment liquid (Soudham et al., 2011). The results obtained with borohydride were compared to those obtained with dithionite (Fig. 4). The results indicate that addition of sodium borohydride to pretreatment liquid did not improve enzymatic hydrolysis of cellulose in the presence of pretreatment liquid as sodium dithionite does. Furthermore, the experiments show that sodium borohydride had a negative effect on the enzymatic hydrolysis in the absence of hydrolysate, which is expected considering the results obtained with other reducing agents (Soudham et al., 2011). In the presence of pretreatment liquid, addition of sodium borohydride was not clearly negative as in the experiments with buffer without inhibitors (Fig. 4), but rather neutral. Sodium dithionite showed positive effects both at 15 and 30 mM (Fig. 4). Sodium borohydride would be expected to have a positive net effect in a simultaneous saccharification and fermentation (SSF), as a result of a neutral effect on enzymatic hydrolysis and a positive effect on the microbial fermentation. However, the reaction mixture would differ since an SSF is based on a slurry that contains both a liquid and a solid phase. The efficiency of sodium borohydride with regard to treatment of slurries remains to be investigated.

3.3. Treatment of model inhibitors

In order to better understand the chemical effects of sodium borohydride on fermentation inhibitors, an experiment was conducted with four selected aromatic compounds and one furan aldehyde. The aromatic compounds included one aldehyde, one carboxylic acid, and two quinones. *p*-Benzoquinone is known to be very toxic to *S. cerevisiae* (Larsson et al., 2000). Table 3 shows the results from analyses using UHPLC-ESI–TOF-MS. The "treated/untreated" values displayed in Table 3 are the relative concentrations after treatment with sodium borohydride in relation to the untreated sample. The quantification was based on the mass chromatogram when that was possible and on the UV chromatogram when it was not feasible to use the mass chromatograms. The peak areas given in Table 3 are based on the deprotonated ion of each compound in the samples, as well as on formic acid adducts and dimers when those were present in the MS spectra (coniferyl aldehyde and ferulic acid). Analysis of the results with UHPLC-ESI–TOF-MS shows that the model fermentation inhibitors were strongly affected by sodium borohydride when added in equivalent amounts, with the exception of ferulic acid, which was not affected. Ferulic acid was affected by treatment with sulfur oxyanions (Cavka et al., 2011) indicating that sodium borohydride, as expected, affects inhibitors differently. The reactions with coniferyl aldehyde and *p*-benzoquinone were exhaustive, while 2,6-dimethoxybenzoquinone and furfural reacted to the extent that 14% of each compound remained (Table 3). Resulting peaks of the compounds which were treated were difficult to detect on the mass spectrometer, which suggests that they were difficult to ionize with the electrospray, which is considered a moderate to soft ionization technique. In order to better understand the mechanism behind the treatment, a UV spectrum was used to identify the resulting compounds. Coniferyl aldehyde and the peak resulting after borohydride treatment are shown in Fig. 5. The resulting compound was identified as coniferyl alcohol. Similar reactions occurred for other reacting compounds. *p*-Benzoquinone was reduced to hydroquinone, while furfural was reduced to furfuryl alcohol.

Larsson et al. (2000) examined the toxic effect on yeast of 20 different aromatic compounds including compounds that were used in this study, and some of the resulting compounds that arise after treatment with sodium borohydride. While *p*-benzoquinone completely inhibited yeast growth and ethanol production already at a concentration of 0.02 g/L (0.2 mM), the corresponding alcohol, hydroquinone, had little or no negative effect on growth or ethanol production even at a concentration of 1 g/L (9 mM) (Larsson et al., 2000). The same trend was observed for coniferyl aldehyde and coniferyl alcohol, as coniferyl aldehyde had a much stronger negative effect on yeast growth and ethanol productivity than the alcohol (Larsson et al., 2000). Thus, the reactions observed with sodium borohydride should result in detoxification.

The chemical effect that sodium borohydride treatment has on fermentation inhibitors evidently differs from that of sulfur oxyanions. Cavka et al. (2011) showed that both sodium dithionite and sodium sulfite work through sulfonation of fermentation inhibitors and this results in decreased reactivity as well in a strong hydrophilization, as the sulfonate group is charged at relevant pH values. As expected, the strong hydrophilization associated with sulfonation is not seen with the treatment of the same compounds with sodium borohydride (Fig. 5). Even though coniferyl alcohol elutes earlier than coniferyl aldehyde (Fig. 5), it is not eluted directly with the front as a strongly hydrophilic sulfonated compound would. It is interesting that despite the different mechanisms of sodium borohydride and sulfur oxyanions, the detoxification effects on yeast seem to be very similar. Our study clearly shows that sodium borohydride is equally efficient to sodium dithionite and superior to sodium sulfite when compared at the same concentrations (Fig. 3). This suggests that decreased reactivity

Table 3
Relative concentrations (%) of aromatic compounds and furfural before and after treatment with $NaBH_4$.[a]

Detected ions $[M-H]^-$ and $[M-H+HCOOH]^-$ and $[M_2-H]^-$	Treatment[a]	Average peak area	Standard deviation	Treated/untreated (%)
Coniferyl aldehyde	Untreated	3.5×10^5	3.4×10^4	100
177.05 + 223.05 + 355.11	$NaBH_4$	438	21	<0.01
Ferulic acid	Untreated	2.2×10^4	4.2×10^3	100
193.05 + 239.06	$NaBH_4$	2.2×10^4	1.1×10^3	100
p-Benzoquinone	Untreated	957	70	100
UV: 221.90, 289.90 nm	$NaBH_4$	N.D.[b]	N.D.	<0.01
2,6-Dimethoxybenzoquinone	Untreated	5.2×10^3	476	100
UV: 289.90 nm	$NaBH_4$	746	11	14
Furfural	Untreated	7.9×10^3	174	100
UV: 227.90, 276.90 nm	$NaBH_4$	N.D.	N.D.	14

[a] Duplicates of each sample were analyzed.
[b] N.D. = not detected.

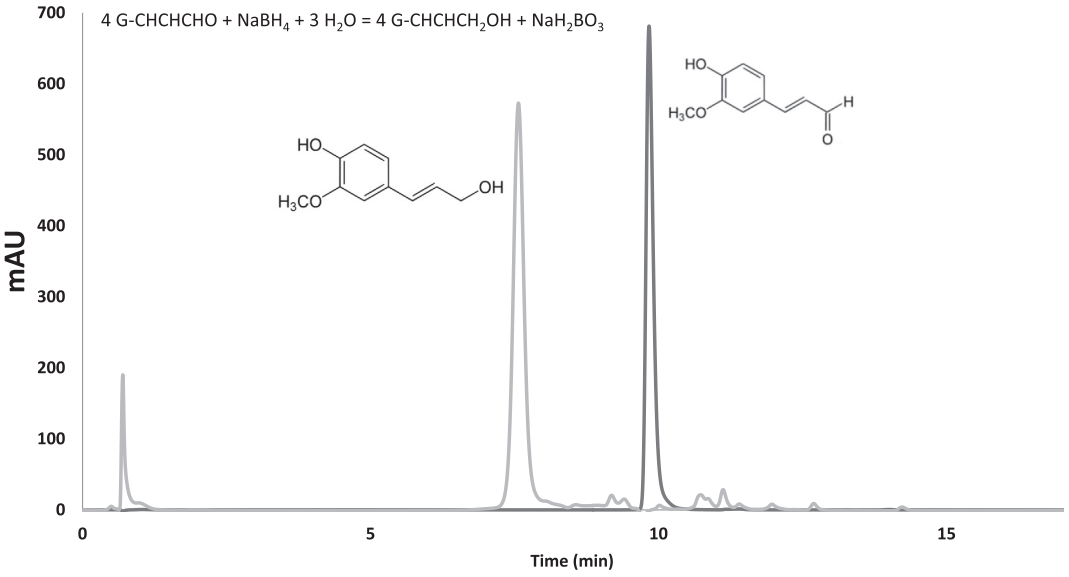

Fig. 5. UV–DAD detection of coniferyl aldehyde (dark grey, signal at 330 nm, elution after 9.9 min) and coniferyl alcohol (light grey, signal at 217 nm, elution after 7.6 min) produced by treatment of coniferyl aldehyde with sodium borohydride. The stoichiometry of the reaction is indicated in the figure (G denotes guaiacyl moieties, i.e. 4-hydroxy-3-methoxyphenyl groups).

is very important for decreasing the toxicity of fermentation inhibitors, while the hydrophilicity is less important. As dithionite improves enzymatic hydrolysis in the presence of pretreatment liquid, while borohydride does not, the situation is probably reversed for enzyme inhibition. In this case, the strong hydrophilicity brought about by sulfonation seems to be important. This suggests that hydrophobic interactions are important in inhibition of enzymatic hydrolysis.

4. Conclusions

This investigation shows that detoxification with sodium borohydride can be as effective as when sulfur oxyanions, such as dithionite, is used. A threefold increase in ethanol productivity and balanced ethanol yield was observed for sodium borohydride and sodium dithionite detoxified hydrolysates compared to the untreated control. For sodium sulfite, there was a twofold improvement. Sodium borohydride can be added in high concentrations (up to about 50 mM) to the fermentation vessel without affecting the fermenting microorganism negatively, which makes is suitable for chemical in situ detoxification. Effective detoxification reactions occured under mild conditions, i.e. room temperature and pH 6, which contribute to making sodium borohydride technically useful for detoxification. The results also indicate that comparisons of the actions of different reducing agents are useful for elucidating differences between inhibition of enzymatic and microbial biocatalysts.

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