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Treatment with Lignin Residue

A Novel Method for Detoxification of Lignocellulose Hydrolysates

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Abstract

Acid hydrolysis of lignocellulose to hydrolysates intended for production of fuel ethanol results in the formation of byproducts in addition to fermentable sugars. Some of the byproducts, such as phenolic compounds and furan aldehydes, are inhibitory to the fermenting microorganism. Detoxification of the hydrolysates may be necessary for production of ethanol at a satisfactory rate and yield. The lignin residue obtained after hydrolysis is a material with hydrophobic properties that is produced in large amounts as a byproduct within an ethanol production process based on lignocellulosic raw materials. We have explored the possibility of using this lignin residue for detoxification of spruce dilute-acid hydrolysates prior to fermentation with *Saccharomyces cerevisiae*. Three dilute-acid hydrolysates of spruce were treated with lignin residue, which in all cases resulted in improved fermentability in terms of productivity and yield of ethanol. The effect was improved by washing the lignin before treatment, by using larger amounts of lignin in the treatment, and by performing the treatment at low temperature. Treatment with the lignin residue removed up to 53% of the phenolic compounds and up to 68% of the furan aldehydes in a spruce dilute-acid hydrolysate. A larger fraction of furfural was removed compared to the less hydrophobic 5-hydroxymethylfurfural.

Index entries: Ethanol; lignocellulose; inhibitors; detoxification; lignin; *Saccharomyces cerevisiae*.

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Introduction

Increased environmental concern worldwide has pointed to the need for alternatives to the use of fossil fuels for transportation. Using fuel ethanol produced from renewable resources does not result in a net contribution of carbon dioxide to the atmosphere. Ethanol can also serve as an oxygenate in fuel. Sugarcane juice and starch-based materials, such as grains, are major feedstocks for the production of fuel ethanol (1). Lignocellulosic materials, such as wastes from forestry, could provide an abundant alternative raw material for fuel ethanol production if all the challenges associated with hydrolysis and fermentation processes can be overcome. Dilute-acid hydrolysis is known to be an efficient method to hydrolyze lignocellulose polysaccharides to fermentable sugars (2), which then can be metabolized to ethanol by using, e.g., the yeast *Saccharomyces cerevisiae*.

During the harsh conditions of acid hydrolysis, compounds that are inhibitory to *S. cerevisiae* are also formed, resulting in a lower fermentability of the hydrolysate. Inhibitory phenolic compounds are formed by the degradation of lignins *(3–8)*. Sugars are degraded to inhibitory furan aldehydes—hexoses to 5-hydroxymethylfurfural (5-HMF) and pentoses to furfural *(9,10)*. The fermentation inhibitors also include aliphatic acids, such as acetic, formic and levulinic acid *(11,12)*.

Owing to the presence of these fermentation inhibitors, detoxification of the hydrolysate before fermentation may be necessary to obtain a reasonable ethanol production. A variety of approaches have been taken to detoxify lignocellulosic hydrolysates. Some of the detoxification methods evidently rely on hydrophobic extraction. Extraction of an aspen wood hydrolysate with ethyl acetate *(13)* has been shown to completely remove furfural, *p*-hydroxybenzoic acid, and vanillin from the hydrolysate, and the extraction also decreased the level of acetic acid. Subsequent fermentation with *Pichia stipitis* showed that the ethyl acetate extraction had improved the fermentability. Recently, an uncharged chromatography resin (XAD-8) was shown to remove phenolic compounds, furfural, and 5-HMF from a spruce acid hydrolysate *(14)*. The XAD-8 resin did not affect the concentrations of the polar compounds acetic acid and glucose. This demonstrated that solid-phase extraction could serve as a method to remove phenolic compounds and furan aldehydes by hydrophobic interactions.

Liquid-liquid extraction and treatment with chromatography resins are both efficient methods for improving the fermentability of hydrolysates, but they also have drawbacks. Liquid extraction requires large quantities of solvent, and distillation would be necessary prior to reuse. Chromatography resins are costly, and it would be an advantage to use alternative materials with comparable hydrophobic properties. One such alternative is lignin, a cheap and plentiful waste product. Lignins are complex aromatic macromolecules, formed from phenylpropanoid precursors. During acid hydrolysis of lignocellulose, a minor part of the lignin is degraded, forming low molecular weight phenolic compounds. Most of

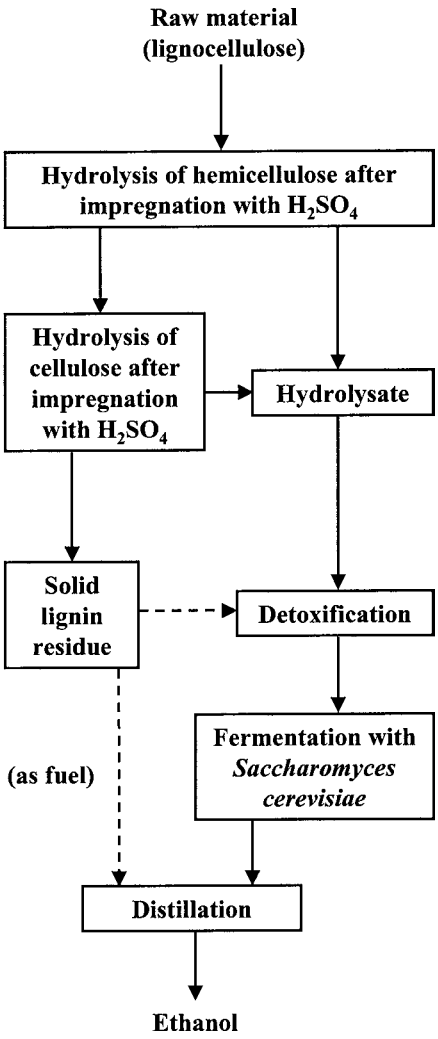

Fig. 1. Flow chart of tentative ethanol production process in which lignin-containing hydrolysis residue is exploited for detoxification, before it is used as fuel.

the lignin is separated as a solid phase after the hydrolysis step and can be dried and used as a fuel for distillation in an ethanol production process. Since lignin is one of the three major components of lignocellulose, the lignin-rich solid residue represents a major byproduct generated within the process. Here, we explore the possibility of taking advantage of the hydrophobic properties of the lignin residue and using it for detoxification of the hydrolysate before it is used as a solid fuel in the distillation (Fig. 1). The effect of lignin treatment on the fermentability of acid hydrolysates of spruce was investigated, as well as the effect on the concentrations of phenolic compounds, furfural, and 5-HMF in the hydrolysates. Treatment of hydrolysates with lignin under different conditions, such as pH and temperature, was also examined.

Table 1
Chemical Composition of Three Different Spruce Hydrolysates

	Fermentable sugar (g/L)	5-HMF (g/L)	Furfural (g/L)	Phenols (g/L)
Hydrolysate 1	32.2	3.7	0.8	3.7
Hydrolysate 2	39.3	5.2	0.7	4.1
Hydrolysate 3	38.3	2.0	0.6	3.3

Table 2
Frames of Factorial Design Experiment

Variable	Low level	High level
pH	2.0	5.5
Temperature (°C)	4	37
Amount of lignin (g/L)	30	180

Materials and Methods

Hydrolysates

Three hydrolysates (hydrolysate 1, 2, and 3), prepared at different occasions and using different conditions, were used for investigating the effect of treatment with lignin residue. The hydrolysates as well as a solid residue were supplied by Robert Eklund, Mitthögskolan, Örnsköldsvik, Sweden, using methods previously described *(8,15)*. All the hydrolysates were made using chipped spruce as the raw material and sulfuric acid as the impregnation agent. Hydrolysates 1 and 2 were prepared by one-step hydrolysis and hydrolysate 3 by two-step hydrolysis. Table 1 gives the chemical composition of the hydrolysates.

Treatment with Lignin Residue

The lignin residue was washed with distilled water or, alternatively, with 1 *M* KOH followed by distilled water. In either case, the wash was continued until the water used for washing had a neutral pH and no color. Various amounts of the spruce lignin residue were added to the hydrolysates. The treatment was performed for 1 h with stirring in sealed flasks. The samples were thereafter filtered or centrifuged to remove particles prior to analytical measurements and fermentations.

Factorial Design

To investigate the influence of pH, temperature, and the amount of added lignin, an experimental design was made with the MODDE 4.0 software (Umetri, Umeå, Sweden). To cover the area of interest with as few experiments as possible, 17, all variables were changed simultaneously, within the frames given in Table 2, and the influences of the factors on the response were investigated.

Analyses of Hydrolysates

The initial concentrations of fermentable sugars were determined with high-performance anion-exchange chromatography using methods previously described *(14)*. The concentrations of 5-HMF and furfural in the hydrolysates were determined using reversed-phase high-performance liquid chromatography (HPLC). A Gynkotek system 480 equipped with a diode array detector, UVD 340S (Gynkotek, Germering, Germany), was used. The samples were diluted in distilled water and injected on a C-18 column ODS-AL (YMC Milford, MA), eluted with a gradient of 3–100% (v/v) acetonitrile and 0.025% (v/v) trifluoroacetic acid using a flow rate of 0.8 mL/min. Syringic acid was used as the internal standard.

The total amounts of phenols in the hydrolysate were estimated with a spectrophotometric method based on the Folin-Ciocalteau reagent (Sigma, Steinheim, Germany). The samples were diluted 25 times with distilled water and 1 mL was transferred to a 50-mL volumetric flask to which 3 mL of the Folin-Ciocalteau reagent and 30 mL of distilled water were added and mixed thoroughly. After 5–8 min, 10 mL of 20% (w/v) sodium carbonate solution was added and the volume was adjusted to 50 mL. The mixture was stirred for 2 h and then the absorbance at 760 nm was measured. The amount of phenols was determined from a calibration curve based on vanillin, since it was the most abundant phenol in the hydrolysates.

Fermentation

The fermentability of the hydrolysates before and after detoxification was evaluated in 25-mL fermentors using an operating volume of 20 mL. The fermentors were sealed with rubber stoppers and equipped with cannulas for carbon dioxide removal. The fermentations were performed with 95% (v/v) hydrolysate 1 or 2 and 65% (v/v) hydrolysate 3. The hydrolysate was supplemented with nutrients to the following final concentrations: 1 g/L of yeast extract, 0.5 g/L of $(NH_4)_2HPO_4$, 0.025 g/L of $MgSO_4 \cdot 7H_2O$, 1.38 g/L of NaH_2PO_4, and glucose so that the total amount of fermentable sugars (glucose and mannose) reached 35 g/L. Prior to fermentation, untreated as well as detoxified hydrolysates were adjusted to pH 5.5 using NaOH. An inoculum of 2 g/L (dry wt) of *S. cerevisiae* (baker's yeast; Jästbolaget AB, Rotebro, Sweden) was used in all fermentations. The fermentations were performed for 24–48 h at 30°C with stirring (200 rpm) and under oxygen-limited and nonsterile conditions. All fermentations were performed in duplicate, and control fermentations with 35 g/L of glucose and nutrients as already described were run together with the hydrolysate fermentations. Samples of 100 μL for HPLC analyses were taken at the beginning of the fermentations and then after 2, 4, 6, 8, 12, and 24 h. From the fermentations carried out for 48 h, samples were withdrawn also after 36 and 48 h.

Table 3
Ethanol productivity (Q_{6h}), Formation (C_{24h}), and Yield (Y) for Fermentations with Hydrolysate 1 Treated with 85 g/L of Washed or Unwashed Lignin at pH 2.0 and at 2 or 70°C

Treatment	Q_{6h} (g/[L·h])	C_{24h} (g/L)	Y (g/g)
Untreated	0.37	2.39	0.07
Unwashed lignin (70°C)	0.35	2.38	0.07
Unwashed lignin (2°C)	0.45	5.09	0.15
Washed lignin (2°C)	0.62	10.2	0.29
Reference	1.19 ± 0.03	13.1 ± 0.84	0.44 ± 0.01

Analyses of Fermentations

The formation of ethanol during the fermentations was analyzed using HPLC (Waters, Milford, MA). All samples were diluted 40 times and filtered through 0.2-µm membrane filters (Advantec MFS, Pleasanton, CA) prior to injection. Ethanol was determined using an Aminex HPX-87H column (Bio-Rad, Hercules, CA) connected downstream of a Cation-H Refill Cartridge (Bio-Rad). The separation was performed with a flow rate of 0.6 mL/min with 5 m*M* H_2SO_4 as the mobile phase and a column temperature of 45°C. The separated compounds were detected with a refractive index detector, RID-6A (Shimadzu, Kyoto, Japan).

The mean volumetric productivity Q (g/[L·h]) was calculated as the ethanol produced after 6 or 24 h divided by 6 or 24, respectively. Q_{6h} was used for hydrolysate 1 and 2 samples, which fermented more rapidly, and Q_{24h} for hydrolysate 3 samples. The mean volumetric productivity is hereafter referred to as the productivity. The ethanol yield, Y (g/g), was based on the amount of ethanol produced during the fermentation divided by the total amount of fermentable sugars (glucose and mannose) present in the beginning of the fermentation. The formation of ethanol, C (g/L), at the end of the fermentations was measured after 24 h for hydrolysate 1 and 2 and after 48 h for hydrolysate 3.

Results

Effect of Wash and Temperature

Comparison of fermentations with hydrolysate 1 treated at 2°C and pH 2.0 with 85 g/L of either unwashed lignin or lignin washed with water showed a clear increase in fermentability when detoxification was performed with lignin washed with water (Table 3). The productivity increased from 0.45 g/(L·h) for the hydrolysate treated with unwashed lignin to 0.62 g/(L·h) for the hydrolysate treated with washed lignin. Compared to untreated hydrolysate, which showed a productivity of only 0.37 g/(L·h), both treatments improved the ethanol production. That the hydrolysate treated with washed lignin fermented better than hydrolysate treated with

Table 4
Evaluation of Productivity (Q_{6h}), Ethanol Formation (C_{24h}), and Yield (Y) After Treating Hydrolysate 2 with Different Amounts of Lignin Washed with Water or Alkali [a].

Treatment	Q_{6h} (g/L·h)	C_{24h} (g/L)	Y (g/g)
Untreated	0.16	1.25	0.04
Lignin (50 g/L)	0.23	2.21	0.06
Lignin (100 g/L)	0.30	3.38	0.10
Lignin (145 g/L)	0.36	5.72	0.16
Lignin (pH 2.0, 100 g/L)	0.43	6.42	0.18
Alkali-washed lignin (75 g/L)	0.49	7.06	0.20
Reference	1.19 ± 0.03	13.1 ± 0.84	0.44 ± 0.01

[a] Treatments were performed at pH 5.5 and 2°C, unless stated otherwise.

unwashed lignin was also supported by the ethanol yield, which was 0.29 g/g for the treatment with washed lignin compared with 0.15 g/g for the treatment with unwashed lignin. By comparison, fermentation of untreated hydrolysate showed a yield of only 0.07 g/g. The ethanol formed at the end of the fermentations, which was twice as high in the hydrolysate treated with washed lignin compared with unwashed lignin, also supports the increase in fermentability after treatment with washed lignin.

Treatment with lignin resulted in a decrease in the concentrations of furan aldehydes, although there was only a minor difference between treatment with unwashed and washed lignin. The main difference in the effect of the washed lignin was found for the concentration of phenols, which was unchanged when the treatment was performed with unwashed lignin, while it decreased 20% when lignin washed with water was used instead.

The effect of temperature on the detoxification process was studied using the same hydrolysate (hydrolysate 1) detoxified with 85 g/L of unwashed lignin at pH 2.0 and either 2 or 70°C. Treatment at the lower temperature resulted in a higher productivity and a slightly higher ethanol yield (Table 3). The productivity was raised from 0.35 to 0.45 g/(L·h) when the lignin treatment was performed at the lower temperature. Lignin treatment at low temperature (2°C) resulted in an increase in ethanol yield from 0.07 to 0.15 g/g and a doubling of ethanol concentration (Table 3). Lignin treatment at high temperature (70°C) resulted in roughly the same productivity, ethanol yield, and formation of ethanol as that obtained with untreated hydrolysate.

Analyses indicated a decrease in the concentration of furfural at 70°C. This was attributed to evaporation, since the concentration of the less volatile 5-HMF *(8)* was not affected.

Effect of Alkali Wash, pH, and the Amount of Lignin Added

Fermentations of hydrolysate 2 showed a productivity of 0.49 g/(L·h) for hydrolysate treated with alkali-washed lignin compared with 0.30 g/(L·h) for the hydrolysate treated with water-washed lignin (Table 4). The

ethanol yield increased from 0.10 to 0.20 g/g when alkali-washed lignin was used for detoxification instead of water-washed lignin. By comparison, untreated hydrolysate gave a productivity of only 0.16 g/(L·h) and an ethanol yield of only 0.04 g/g. The increased fermentability was supported by a higher formation of ethanol in hydrolysate treated with alkali-washed lignin.

The content of both furans and phenols decreased after treatment with water- and alkali-washed lignin. Alkali-washed lignin resulted in better removal of phenols and 5-HMF than water-washed lignin, while there was no clear difference with respect to furfural.

Hydrolysate 2 was treated with 100 g/L of water-washed lignin at 2°C and at pH 2.0 or 5.5 to investigate the influence of pH on the detoxification method. The fermentations showed a higher productivity (0.43 g/[L·h]) when the lignin treatment was performed at pH 2.0 compared with 5.5 (0.30 g/[L·h]) and also when compared to untreated hydrolysate which only gave a productivity of 0.16 g/(L·h). The same was found for the yield, which increased from 0.04 g/g for untreated hydrolysate to 0.10 g/g for treatment with lignin at pH 5.5 and 0.18 g/g for treatment at pH 2.0 (Table 4).

The amount of added lignin was studied in fermentations using hydrolysate 2 treated at 2°C and pH 5.5 with 50, 100, or 145 g/L of water-washed lignin. When a higher amount of lignin was used for detoxification, a higher productivity was obtained (Table 4). The productivity after 6 h increased with increasing amounts of lignin- from 0.16 (untreated hydrolysate) to 0.23 (50 g/L), 0.30 (100 g/L), and 0.36 g/(L·h) (145 g/L). The corresponding ethanol yields showed a similar increase, from 0.04 g/g for untreated hydrolysate to 0.06 (50 g/L), 0.10 (100 g/L), and 0.16 g/g after treatment with 145 g/L of lignin. The concentrations of the remaining analyzed compounds decreased with increasing amount of lignin added.

Factorial Design Experiment

MODDE, a statistical program for experimental planning and optimization, was used to design a factorial experiment with lignin detoxification of hydrolysate 3. Three parameters were studied: pH, temperature, and amount of lignin added. A set of 17 experiments with different conditions (see Table 2) was designed and performed. The amount of lignin used had a more substantial impact on the concentrations of phenols and furaldehydes than either temperature or pH, with a high amount of lignin removing more inhibitors (Fig. 2). Within the interval studied, the pH did not have any statistically significant effect on the decrease in the concentration of phenols, furfural, or 5-HMF (data not shown). Low temperature contributed to an increased removal of both 5-HMF and furfural (Fig. 2) but did not have any significant effect on phenols. Treatment of the hydrolysate under the most beneficial conditions, a large amount of lignin (180 g/L) and a low temperature (4°C), resulted in removal of 46% 5-HMF and 68% furfural. When lignin was added to 180 g/L, 53% of the phenols was removed. The

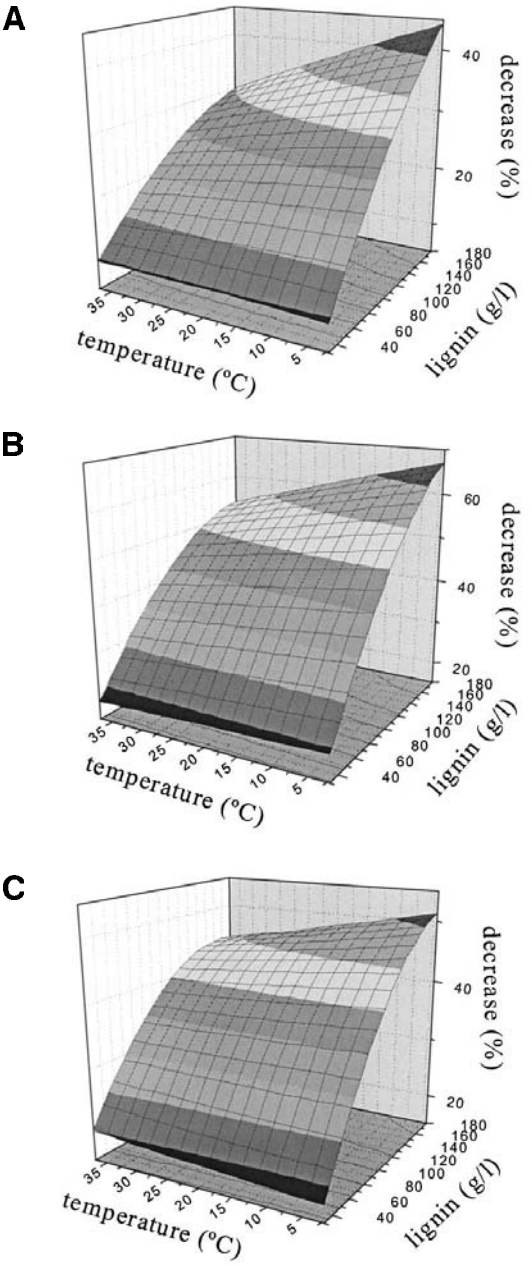

Fig. 2. Results from factorial design experiment with treatment of hydrolysate 3 at different temperature and with different amounts of lignin. Response surfaces for **(A)** 5-HMF, **(B)** furfural, and **(C)** phenols are shown.

degree of removal of phenolic compounds and furan aldehydes as an effect of varying concentrations of lignin could be predicted on the basis of the results, as shown in Fig. 3. The curve for phenols in Fig. 3 should represent the average for the many different phenolic compounds that are present in the hydrolysate and detected by the Folin-Ciocalteau method.

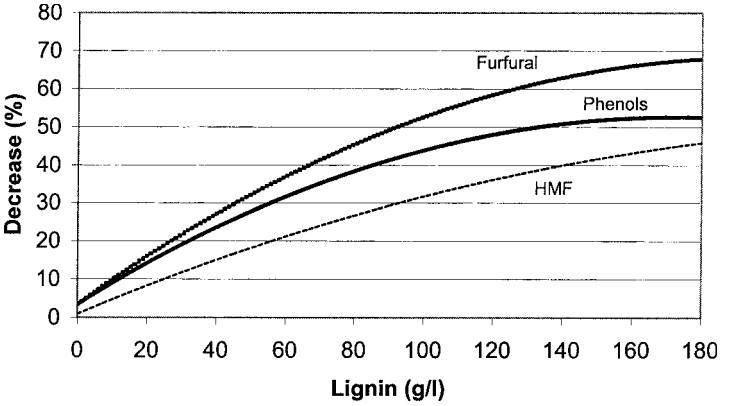

Fig. 3. Prediction of effect of lignin treatment on content of phenols and furan aldehydes. The prediction is based on the results from the factorial design experiment using hydrolysate 3 at pH 5.5 and 4°C.

Table 5
Ethanol Productivity (Q_{24h}), Ethanol Formation (C_{48h}), and Yield (Y) for Fermentations of Hydrolysate 3 Treated with Different Amounts of Lignin

Treatment	Q_{24h} (g/[L·h])	C_{48h} (g/L)	Y (g/g)
Untreated	0.11	2.68	0.08
Lignin (20 g/L)	0.13	3.72	0.12
Lignin (50 g/L)	0.15	4.76	0.15
Lignin (100 g/L)	0.25	12.8	0.44
Reference	0.58	14.3	0.46

The Effect of Amount of Lignin Added on Fermentability and Inhibitors of Hydrolysate 3

The amount of lignin added was shown to be the main parameter affecting the removal of phenols, 5-HMF, and furfural. Therefore, treatment of hydrolysate 3 with different amounts of lignin was also evaluated in fermentations. Hydrolysate 3 was treated at 4°C and pH 5.5 with 20, 50, or 100 g/L of lignin washed with water. Productivity increased with increasing amount of lignin used for detoxification (Table 5). The untreated hydrolysate had a productivity of 0.11 g/(L·h), while treatment with lignin increased this to 0.13 (20 g/L), 0.15 (50 g/L) and 0.25 g/(L·h) (100 g/L). The ethanol yield also increased with increasing amounts of lignin used, from 0.08 (untreated hydrolysate) to 0.12 (20 g/L), 0.15 (50 g/L), and up to 0.44 g/g (100 g/L of lignin). The same trend was found for the amount of ethanol formed after 48 h of fermentation.

Removal of phenols, 5-HMF and furfural increased with increasing amounts of lignin, as predicted by the model (Fig. 3). The addition of 20 g/L of lignin decreased the phenols by 16%, 5-HMF by 4%, and furfural by 13%.

An increase to 50 g/L of lignin resulted in a 24% decrease in phenols, 13% decrease in 5-HMF, and 30% decrease in furfural, while 100 g/L of lignin removed 36% of the phenols, 27% of the 5-HMF, and 49% of the furfural.

Discussion

Treatment of acid hydrolysates of spruce with lignin obtained as a hydrolysis residue within the ethanol production process was shown to result in improved fermentability and affords a new way to deal with fermentation inhibitors in lignocellulose hydrolysates. Previous results with detoxification using a material (XAD-8) displaying hydrophobic properties *(14)* supported the idea that solid-phase extraction should be a feasible approach to treat lignocellulose hydrolysates prior to ethanolic fermentation. Possible advantages of using residual lignin rather than chromatography resins, such as XAD-8, for detoxification by solid-phase extraction include the following: the lignin is derived from a cheap and abundant raw material; it is produced as a byproduct within the ethanol production process; and after use for detoxification purposes, it can still be employed as a solid fuel for the distillation process together with the inhibitors that were extracted from the hydrolysate.

The results showed that washing the lignin was necessary to achieve a material that could efficiently extract fermentation inhibitors with hydrophobic properties, such as phenolic compounds and furan aldehydes, from the hydrolysates. Probably, the lignin residue has already to some extent removed phenolic compounds and furan aldehydes from the hydrolysate before they are separated (Fig. 1). If unwashed lignin is used, the sites of interaction may already be saturated to a high degree, but become available again for interaction with phenolic compounds and furan aldehydes after washing. The results furthermore indicate that if alkali is included in the washing step, a more efficient material for detoxification can be obtained. Treatment of spruce acid hydrolysates with alkali is in itself a rather efficient detoxification method for spruce acid hydrolysates *(8)*, and in order to keep the effects apart and focus on the effect of the lignin, which was the objective of our study, all treatments of hydrolysates were performed at acidic pH.

Since phenolic compounds turn into charged phenolates at higher pH, hydrophobic interactions with the lignin may consequently be more efficient at acidic pH. Since the pH of the hydrolysates initially was about 2.0 and the pH of the fermentation was set to 5.5, the effect of varying the pH between 2.0 and 5.5 was examined in the factorial design experiment. It can be concluded that there was no major effect on the removal of phenolic compounds and furan aldehydes within this pH interval. Better fermentability of hydrolysate 2 after treatment at pH 2.0 compared with pH 5.5 can be explained by phenolic acids, which are uncharged at pH 2.0 but to a large extent are charged at pH 5.5 *(16)*. A separate series of analytical experiments showed better removal of phenols at pH 2.0 than pH 5.5.

The temperature should be kept low during lignin detoxification, as was shown by the factorial design experiment (Fig. 2). In that experiment, the temperature was varied between 4 and 37°C, but the impact of the temperature on the concentrations of phenols and furan aldehydes was still rather limited. The experiment with hydrolysate 1, in which the temperature was either 2 or 70°C, showed clearly that the fermentability was better after treatment at the lower temperature, which agrees with the results from the factorial design experiment. Because removal of inhibitors from hydrolysate is most efficient at lower temperature, this should also be considered when the solid fraction is separated from the liquid fraction after hydrolysis steps.

Treatment with lignin showed a more obvious effect on furfural than on total phenols, which, in turn, were more efficiently removed than 5-HMF (Fig. 3). Since the removal of the compounds can be expected to be based on hydrophobic properties, it is an expected result that furfural was removed more efficiently than 5-HMF, which has a more polar character owing to its hydroxyl group. The phenolics are a very heterogeneous group and separate phenolics differ with respect to hydrophobicity. The pH will also affect the charge of phenolic carboxylic acids and hence the hydrophobicity.

The efficiency of the treatment with lignin was found to be highly dependent on the amount added. Besides the wash of the lignin, this was the most apparent way to increase the efficiency of the removal of phenols and furan aldehydes as well as to improve the fermentability. Both these findings support that it is the number of sites on the solid material that is available for interaction with inhibitors that determines the efficiency of the detoxification. Methods to increase the content of available interaction sites on the material used for the solid-phase extraction are therefore of great interest to make the use of lignin as a detoxifying agent for lignocellulose hydrolysates more attractive. The fact that the treatment with lignin residue increased the fermentability for three different hydrolysates shows that this technique of absorption on lignin has great potential to be used in the process of fuel ethanol production using different types of hydrolysates.

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