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The Use of Steamed Hemicellulose as Substrate in Microbial Conversions

Scientific Note

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ABSTRACT

The suitability of acid- and enzymatically hydrolyzed birch hemicellulose as biotechnical raw material was studied using *Gluconobacter oxydans, Fusarium oxysporum,* and *Candida utilis* for production of xylonic acid, ethanol, and SCP, respectively. The fermentabilities of both hydrolyzates were rather similar and inhibition was evident in all cases at xylose concentrations of 25–30 g/L and higher. Potential identified fermentation inhibitors were the lignin-derived compounds sinapyl alcohol, coniferyl alcohol, vanillin, and syringaldehyde and the sugar degradation products furfural and 5-hydroxymethyl furfural.

Index Entries: Hemicellulose hydrolyzate; *Gluconobacter oxydans; Candida utilis; Fusarium oxysporum;* fermentation inhibitors.

INTRODUCTION

The use of lignocellulosic materials, such as wood and agricultural crop residues, as a renewable source of chemicals has received considerable interest in recent years. Cellulose is most economically utilized in fiber, but hemicellulose could serve as a carbon source in biotechnical processes.

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Wood usually contains 20–30% hemicellulose and the main type in hardwoods is D-xylan. Microorganisms can convert xylose to various products including alcohols, organic acids, ketones, polyols, or single cell protein. However, most reports concerning biotechnical conversion of xylose deal with the fermentation of pure xylose. The use of practical raw materials, such as spent sulphite liquor or various hemicellulose hydrolyzates, is complicated by the presence of inhibitory compounds (soluble lignin- and sugar degradation products) in the hydrolyzates. Therefore, hemicellulose hydrolyzates are not easily utilized by microorganisms. The tolerances of different microbial strains towards potential inhibitors found in hemicellulose hydrolyzates also vary significantly (*1*). Various methods for circumventing the inhibitory effect have been proposed (*2–6*).

In the steaming process, hemicellulose becomes water soluble and can subsequently be extracted with water. However, it is only partially hydrolyzed in steaming and monomeric sugars comprise only a minor part of the total extracted carbohydrates (*7*). Most xylose-utilizing microorganisms are not hydrolytic and cannot ferment xylo-oligomers. The hemicellulose must therefore be hydrolyzed. Hemicellulose can be hydrolyzed by mild acid hydrolysis or by a mixture of hemicellulases (*8,9*).

The aim of this study was to compare the suitabilities of acid- and enzymatically hydrolyzed birch hemicellulose as raw material for biotechnical conversion by *Gluconobacter oxydans*, *Fusarium oxysporum*, and *Candida utilis*. These organisms have been reported to utilize xylose efficiently for production of xylonic acid, ethanol, and SCP (*1,10*). Attempts were also made to identify potential inhibitory compounds in the hydrolyzates.

METHODS

Microorganisms

Gluconobacter oxydans subsp. *suboxydans* ATCC 621, *Candida utilis* ATCC 9226, and *Fusarium oxysporum* VTT-D-80134 were used for the production of xylonic acid, SCP, and ethanol, respectively. *G. oxydans* was maintained as described previously (*1*). *C. utilis* was maintained on YM-agar slants (Difco 0712). *F. oxysporum* was maintained on oatmeal agar slants (Difco 0552).

Cultivation Media and Conditions

All cultivations were carried out in 250 mL conical flasks containing 50 mL broth. The cultivation temperature was 30°C and the agitation speed was 200 rpm. *G. oxydans* was cultivated in shake flasks as described previously (*1*). The cultivation medium for *C. utilis* contained per liter: 5g $(NH_4)_2HPO_4$, 5g yeast extract, and pure xylose or hemicellulose hydrolyzate in different concentrations, as described in the text. The inoculum was cultivated on medium containing per liter: 3 g malt extract, 3 g yeast extract, 5 g Bacto peptone, and 10 g D-xylose. The cultivation time was 3

d. *F. oxysporum* was cultivated in shake flasks as described previously (*7*). Pure xylose or steamed hemicellulose hydrolyzate was added to the media in concentrations from 5 to 45 g/L, as described in the text.

Hemicellulose Hydrolyzates

Birchwood was steamed at 200°C for 15 min and water extracted (*7*). The extract was hydrolyzed either by *Trichoderma reesei* enzymes at 45°C for 48 h (*11*) or by sulfuric acid. In the acid hydrolysis the pH of the extract was set to 1.0 by adding 95% sulfuric acid (4.9 mL to 1.0 L of extract) and the birch extract was refluxed at 100°C for 4 h. After both hydrolyses the hydrolyzates were filtered through Whatman No. 1 filter paper in order to remove insoluble residues. The pH of the acid hydrolyzate was first adjusted to 4.0 with a 15% slurry of $Ca(OH)_2$ in order to remove the remaining sulphates and the precipitate was removed by filtration. Thereafter, pH was adjusted to pH 6.5 with 5 N NaOH and the hydrolyzate was filtered again. Both hydrolyzates were concentrated by vacuum evaporation.

Analyses

Samples from the cultivations were first centrifuged at 5000 rpm for 15 min. Sugars (xylose and glucose) were analyzed from the supernatant by HPLC as described previously (*1,7*). Acetate was determined enzymatically (Boehringer Test Combination 148 261).

The phenolic compounds were analyzed with HPLC. Separation of phenolic acids was performed on a Bakerbond Octadecyl column (4.6× 250 mm). For the elution two solvents were used: A methanol; B 0.001 *M* phosphoric acid. The elution profile was: 0–2 min 10% A in B; 2–30 min 50% A (linear gradient); 30–40 min 100% A (linear gradient). The flowrate was 1 mL/min. The HPLC-system was equipped with a UV-VIS-diode array detector HP 1040 (Hewlett Packard, Waldbronn, FGR), which allowed simultaneous detection at different wavelengths and measurement of the UV spectrum of each separated compound during the elution. The diode-array detector was set at 280 nm and 240 nm with an optical bandwidth of 8 nm. The detector was combined with an HP 300 work station.

Because steaming extracts are a complex matrix of both hemicellulose and lignin derived compounds, organic acids can be detected from the extracts after removal of the bulk quantity of phenols by XAD-treatment. Separation of aliphatic organic acids was performed on an HPX-87H column (7.8×300 mm) (Bio-Rad, Richmond, CA). The eluent was 5 m*M* sulfuric acid at 0.6 mL/min (isocratic). In some cases 5 m*M* sulphuric acid in 20% acetonitrile was used. The diode-array detector was set at 210 nm (4 nm bandwidth) and 254 nm (10 nm bandwidth). Attenuation was set automatically according to the highest peak.

Different lignin monomers in the crude hemicellulose extract were identified from the derivatized chloroform extract by capillary gas chromatography/mass spectrometry as described by Niemelä and Sjöström (*12*).

Table 1
Composition of Acid and Enzymatic Hydrolyzates after Neutralization[a]

Component	Acid hydrolyzate	Enzymatic hydrolyzate
Xylose	49.2	59.2
Glucose	2.1	2.6
Acetate	15.2	15.5
Oligomers	some	trace

[a]Concentrations are expressed as percent of dry weight.

RESULTS AND DISCUSSION

Acid and Enzymatic Hydrolysis of the Birch Extract

The sugar compositions of acid and enzymatic hydrolyzates of steamed birch hemicellulose were rather similar (Table 1). The acid hydrolyzate had a lower xylose content, which was owing to both residual oligomers and degradation of xylose to furfural. Another difference between the hydrolyses was the removal of part of the phenolics in acid hydrolysis (Fig. 1a). The reaction conditions in acid hydrolysis are harsher than in enzymatic hydrolysis, but it has been suggested that some phenolics and sugar degradation products condense in acidic environment and form humic solids (*13*). In this work, these compounds were evidently separated by subsequent filtration after hydrolysis.

Fermentation of the Hemicellulose Hydrolyzates

The acid and enzymatic hydrolyzates were first concentrated to equal xylose concentrations. Cultivations were then carried out on dilutions of this material using *Fusarium oxysporum, Candida utilis,* and *Gluconobacter oxydans* (Figs. 2–4). All the microorganisms tested consumed pure xylose efficiently in these concentrations. When the hydrolyzates were used as carbon source, however, the fermentability was poor. The inhibition was evident at all xylose concentrations above 20 g/L (Figs. 3–4). Rapid decrease of fermentability has been observed previously with *G. oxydans* (*1*). The *Candida* yeast had the highest tolerance toward the inhibitors in the hydrolyzates (Fig. 2). This strain could grow on the hydrolyzates up to xylose concentrations of 30 g/L without major inhibition.

Enzymatically hydrolyzed hemicellulose was somewhat more suitable as carbon source for *G. oxydans* and *F. oxysporum* (Figs. 3–4). The differences in the fermentabilities were, however, not very great. It appears that removal of the phenolic compounds in acid hydrolysis did not facilitate the fermentation. This could be a result of modification of some residual lignin monomers to more toxic compounds or to formation of new inhibitors by sugar degradation in acidic conditions (*14*).

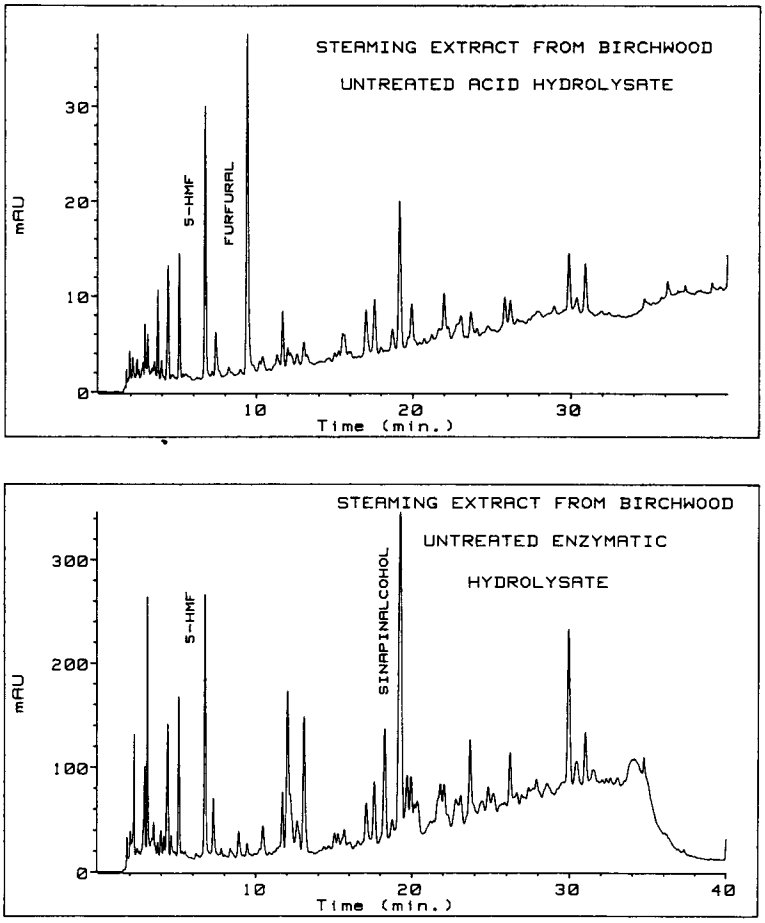

Fig. 1. HPLC-chromatogram of a) phenolic compounds in acid hydrolyzate of steamed hemicellulose. (Nb: the sample was diluted 1:10). b) phenolic compounds in enzymatic hydrolyzate of steamed hemicellulose.

Identification of Possible Inhibitors in the Steaming Extract

One reason for the poor fermentability of the steamed hemicellulose hydrolyzate was probably the presence of soluble lignin degradation products in the hemicellulose extract of steamed birch (Fig. 5). Major lignin monomers identified in the extract were vanillin, syringaldehyde, coniferyl alcohol, and sinapyl alcohol. The hydrolyzates also contained the sugar degradation products furfural and 5-hydroxymethyl furfural (Fig. 1), and organic acids (Fig. 6a). All the peaks in Fig. 6a and b have a spectrum typical of organic acids, with a maximum at 210 nm, and without any signal at 240 nm and higher, although only acetic acid could be identified. The peak at retention time 15 is unknown and was caused by the

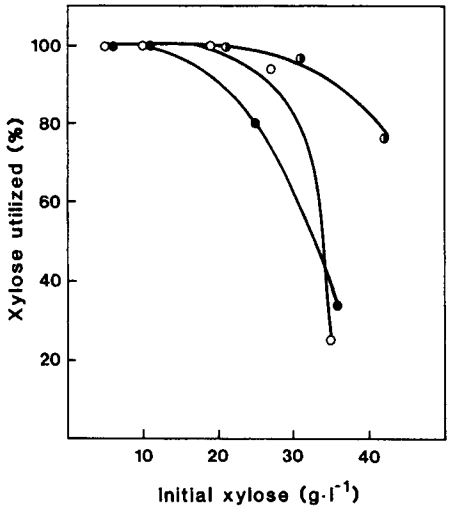

Fig. 2. Cultivation of *Candida utilis* on pure xylose (◑—◑) and on acid (○—○) and enzymatic hydrolyzate (●—●) of steamed hemicellulose. Cultivation time 3d.

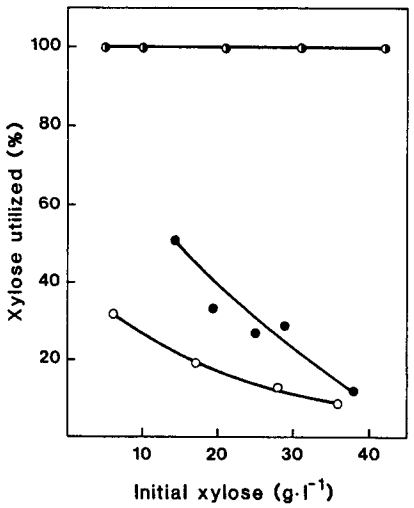

Fig. 3. Cultivation of *Gluconobacter oxydans* on pure xylose (◑—◑) and on acid (○—○) and enzymatic hydrolyzate (●—●) of steamed hemicellulose. Cultivation time 3d.

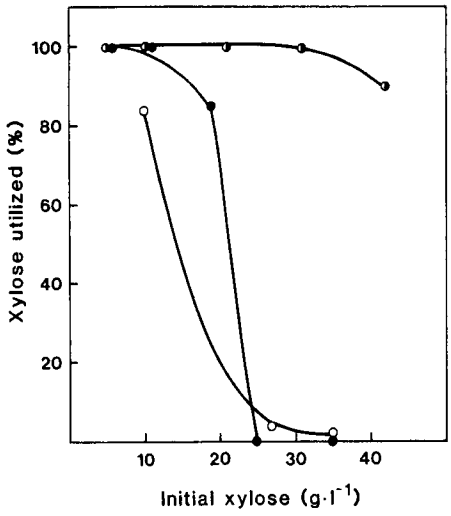

Fig. 4. Cultivation of *Fusarium oxysporum* on pure xylose (◑—◑) and on acid (○—○) and enzymatic hydrolyzate (●—●) of steamed hemicellulose. Cultivation time 6d.

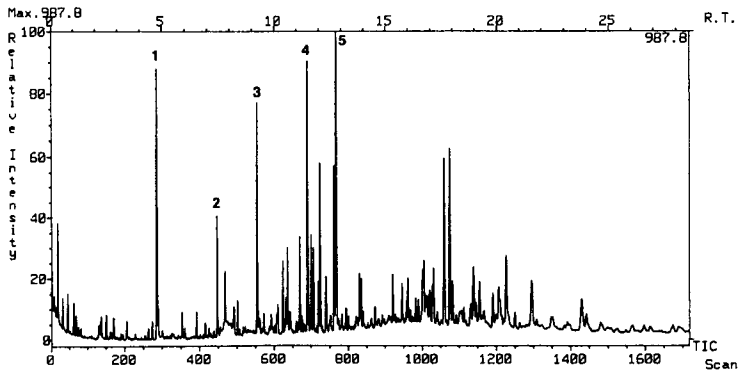

Fig. 5. GLC-spectrum of the chloroform extract of steamed hemicellulose. 1=5-HMF, 2=vanillin, 3=syringaldehyde, 4=coniferylalcohol, 5=sinapyl alcohol.

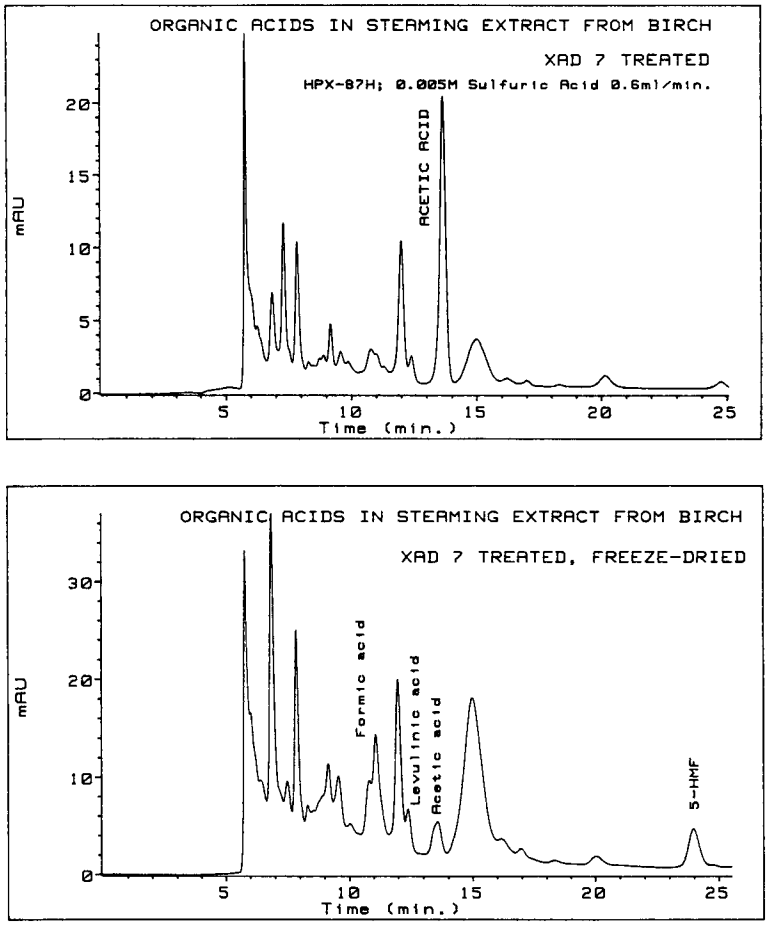

Fig. 6. HPLC-chromatogram of organic acids in a) the enzymatic hydrolyzate of undried steaming extract of birch. b) after freeze drying.

enzyme preparation. Most of the volatile organic acids were removed by freeze drying (Fig. 6b).

Removal of volatiles has been reported to enhance the fermentability of a beech hemicellulose hydrolyzate by *Fusarium* (4). *Fusarium oxysporum* is very sensitive toward acetic acid (*10*) and the acetate of the hydrolyzates (Table 1) may have caused the inhibition. *Gluconobacter* tolerates acetate and furfural relatively well (*1*), but nevertheless this organism was unable to utilize the xylan hydrolyzates efficiently. The inhibitory effects of model compounds of lignin monomers have been studied recently and some proposals for the effects of molecular structure on the toxicity have been made (*15–17*). However, hydrolyzates originating from different sources and processes contain different soluble lignin degradation products (3; Fig. 5). The analysis of the hydrolyzates is imporant in predicting their fermentabilities.

CONCLUSIONS

The results of this study, among others, indicate that the mechanism of inhibition in the hemicellulose hydrolyzate is very complicated. The main fermentation inhibitors were not formed in the final hydrolysis step, but already during steaming. Thus the fermentabilities of acid and enzymatic hydrolyzates were rather similar. Although some probable inhibitors were removed in acid hydrolysis, others were generated. The identification of possible inhibitors and the understanding of their effects on microbial physiology are important in order to develop new methods for circumventing their inhibitory effect.

The concentration of the hemicellulose hydrolyzates is also a critical parameter in their processing by fermentation. The methods for removal of inhibitors must be evaluated in terms of both efficiency and economics. Because the bulk chemicals that could be produced from hemicellulose by fermentation are mainly low-value products, the raw material costs play an important role in process economics.

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