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Utilization of Waste Cellulose

VIII. Enzymatic Hydrolysis of Spruce Bark by Cellulases of *Trichoderma viride*

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ABSTRACT

Native outer, inner and whole bark, and paper mill waste bark have been studied and compared. Extraction with cold and warm water and different chemical pretreatments (H_2SO_4, NaOH, and NaClO) have been used to improve the accessibility of the cellulose fraction of the substrate to the enzymes and hence to increase the yield of formation of glucose.

Index Entries: Bark, inner, outer, whole; bark, reactivity with warm water, cold water; saccharification; enzymatic hydrolysis; *Trichoderma viride*.

INTRODUCTION

Bark is the layer external to the cambium. It amounts to 10–15% of the total weight of the tree. There is a large quantity of waste bark. It is usually burnt; a small amount of it is used in horticulture. The use of bark as a raw material for the production of tannins and other chemicals is decreasing steadily. An exploratory study was thus undertaken to use bark either as a source of fermentable hexoses or pentoses or as a source of degradable aromatic compounds for the production of fine chemicals. The present work concerns the enzymatic hydrolysis of bark by cellulases of *Trichoderma viride*. Different pretreatments were used to improve the

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yield in glucose as done in previous studies of the hydrolysis of spruce and Eucalyptus wood (*1–5*).

Bark is a very complex and badly known material. It consists of outer and inner bark which differ very significantly in anatomy and chemical composition (*6–7*). Thus, special attention was paid to the comparison of these two types of substrates.

Spruce bark is reported to contain about 25–35% of water extractives. Inner bark contains less lignin and more cellulose than outer bark (respectively, 15 and 27% lignin and 23 and 14% cellulose for inner and outer bark). Analysis of the substrates is very difficult owing to their complex composition. Two types of analysis have been performed in the present work: the determination of glucose after quantitative saccharification and the $KMnO_4$ oxidizable content by the K method. The first series of measurements is related to the cellulose content in the substrates but also to the presence of other glucose containing materials (hydrolyzable tannins, pectin, starch, and so on) mainly in the initial unextracted substrates. The same is true for the apparent native lignin content obtained by the K method since many other substances (phenolic acids, tannins, and so on) are also oxidizable by $KMnO_4$. The potential glucose and the apparent lignin content are indispensable nevertheless for comparing the different substrates and the different pretreatments.

EXPERIMENTAL

Substrates

Whole spruce bark (*10*) was stripped manually off a rough spruce timber. Part of the whole bark was separated into inner and outer bark. The quantitative saccharification of these substrates indicates that whole bark contains about 40% of inner bark and 60% of outer bark. Waste whole bark from papermill was also used as substrate. In this case, the bark was mechanically stripped off the timbers after spraying them with cold water for six wk. It then was washed 3 times for 0.5 h in tepid water before use in the laboratory.

Water Extraction

It was performed with stirring either in boiling water for three h or in cold water for 48 h.

Pretreatments

Pretreatment with diluted H_2SO_4 was performed by refluxing 5 g untreated substrate with 250 mL 2% H_2SO_4 for 2 h.

Pretreatments with NaOH were performed by refluxing 5 g untreated substrate in 100 mL NaOH either 0.5% or 5% for 40 min.

Pretreatments with NaClO were performed at room temperature for 0.5 h (2.5 g substrate for 100 mL NaClO solution). Three different ratios of oxidant to substrate were used: 2, 5, and 7 mol/kg. The pH was maintained at a constant value of 8 during the pretreatment.

Weight Loss

Weight loss is measured by weighing the pretreated substrate at constant weight (24 h in an aerated oven at 105°C).

Loss in Cellulose

The quantitative saccharification of the substrate is performed before and after pretreatment. Glucose is analyzed by HPLC a 25-cm length and 0.46-cm id column filled with the anion exchange resin Aminex A-28 in the borate form. A boric-acid and potassium-borate (pH 8.8) buffer is used as mobile phase with flow rate of 0.4 mL/min. Spectrophotometric detection is made at 570 nm, using the *in situ* prepared complex of the sugars with copper 2,2'bicinchoninate.

Quantitative Saccharification

The substrate (250 mg) is dissolved in 3 mL H_2SO_4, 72% (1 h at 30°C). It is diluted with 84 mL water and kept in the oven for 1.5 h at 130°C in septum capped vials. Neutralization of the acid then is performed with solid $Ba(OH)_2$.

Analysis of the Lignin (K Number)

The substrate is milled and the obtained particles (dimension < 200 μm) are suspended in a known amount of $KMnO_4$ (0.01N) in H_2SO_4 (0.4N). After 10 min, the oxidation reaction is stopped by adding an excess KI titrated with $Na_2S_2O_3$. The K number (8) is obtained from the results. This index multiplied by 0.13 gives the percent native lignin in the substrate.

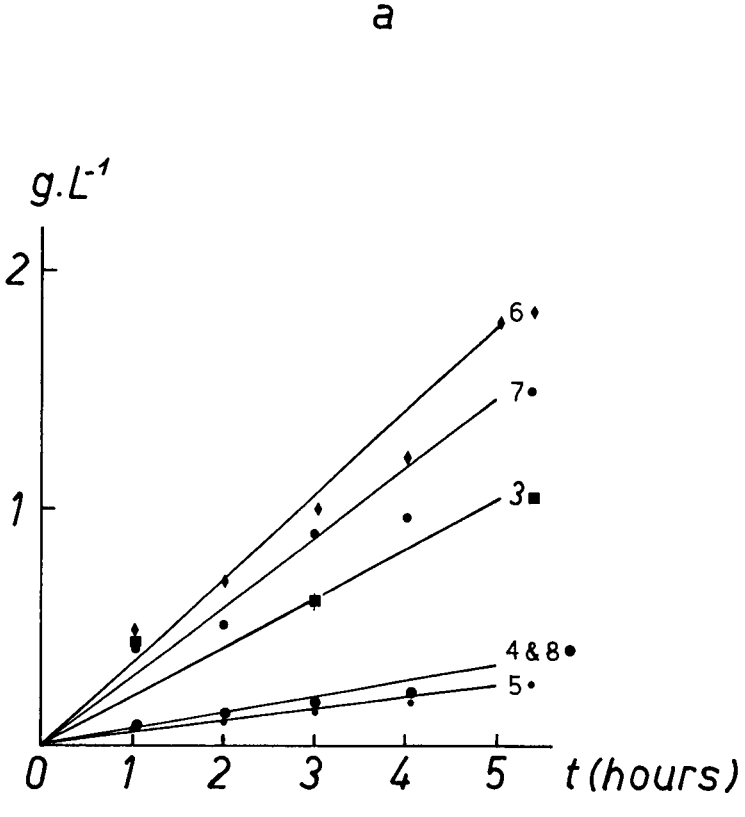
Enzymatic Hydrolysis

The substrate (250 mg) is first soaked for 24 h at room temperature with 9 mL citrate buffer. Cellulases (10 mg) from *Trichoderma viride* (Onozuka R-10 from Kinki Yakult MFG Co., Ltd.) then are dispersed in 1 mL citrate buffer and added to the substrate. The hydrolysis is performed at 45°C. A volume from 10–200 μL is taken after convenient time intervals and analyzed for glucose by the glucose-oxidase method (9) (Boehringer GOD-perid glucose).

RESULTS AND DISCUSSION

Reactivity of Native Outer, Inner, and Whole Bark

Outer, inner, and whole spruce barks have been hydrolyzed in standard conditions (expts. 1–3). The quantity of glucose formed as a function of time is given in Fig. 1 (curve 1–3). As usually observed for other ligno-cellulosic substrates, the quantity of glucose formed first increases and then tends to a limit. This limit is the maximum quantity of glucose available in the experimental conditions of substrate and enzyme concentration used in the present work. It is given in Table 1. Two types of yields have been defined in order to allow comparison between the substrates. The yield Y_I refers to the potential glucose in the substrate which is effectively hydrolyzed by the enzymes. The yields Y_{II} refers to 100 g of the initial substrate. For untreated substrates, Y_I is the quantity of glucose formed after 4 d from 100 g of the initial substrate (a) divided by the potential glucose in 100 g of the initial substrate (b); Y_{II} is the quantity of glucose formed after 4 d from 100 g of the initial substrate (a). For pretreated substrates, Y_I is the quantity of glucose formed after 4 d from 100 g of the pretreated substrate (a) divided by the potential glucose in 100 g of the pretreated substrate (b); Y_{II} is the quantity of glucose formed after 4 d from 100 g of the pretreated substrate (a) multiplied by the percent substrate recovered after the pretreatment (c). The values of Y_I given



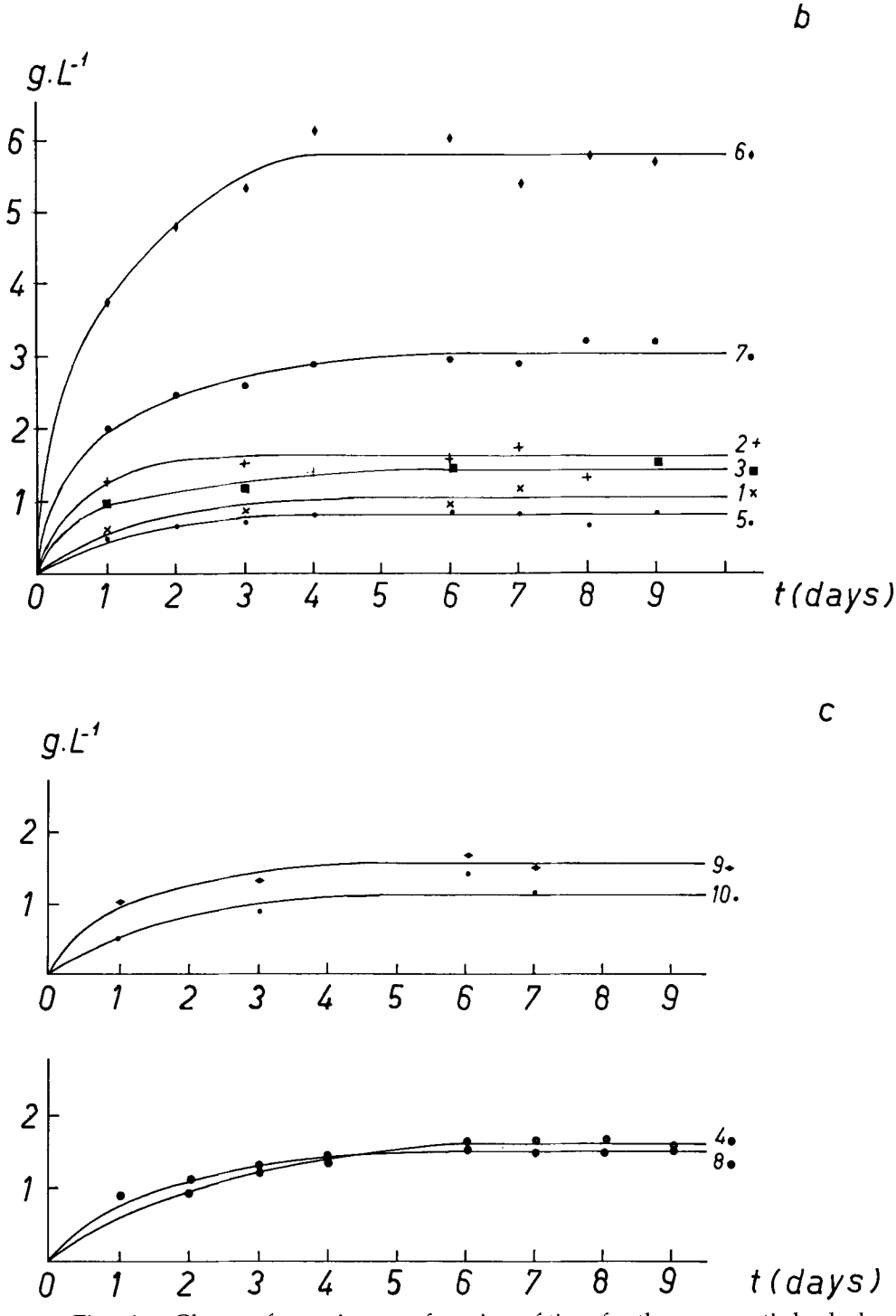


Fig. 1. Glucose formation as a function of time for the enzymatic hydrolysis by cellulases of *Trichoderma viride*, untreated bark: outer (1), inner (2), whole (3), waste paper mill whole (4), boiling water extracted bark: outer (5), inner (6), whole (7), waste paper mill whole (8), cold water extracted bark: inner (9), whole (10). Initial substrate concentration 25 $g.L^{-1}$.

Table 1
Enzymatic Hydrolysis of Spruce Bark by Cellulases of *Trichoderma viride*[a]

Exp	Treatment	Substrate recovered, c %	Potential glucose, b %	Glucose recovered %	Lignin recovered %	Limiting glucose g l^{-1} for 100g l^{-1} subst., 4 d,[a]	Yield I = a/b %	Yield II g/100g, initial bark = a × c
	Untreated							
1	outer	—	21.6	—	—	4.16	19.2	4.2
2	inner	—	34.7	—	—	6.28	18.1	6.3
3	whole	—	26.3	—	—	5.76	21.9	5.8
4	whole (papermill)	—	27.5	—	—	6.48	23.6	6.5
	Boiling water extracted							
5	outer	65	18.2	55	53	3.32	18.2	2.2
6	inner	65	39.2	72	34	22.9	58.4	14.8
7	whole	65	23.4	57	44	12.0	51.3	7.8
8	whole (papermill)	95	26.9	93	100	6.12	22.7	5.8
	Cold water extracted							
9	inner	75	30.7	66	nd[c]	6.24	20.3	4.6
10	whole	75	22	62	nd[c]	4.32	19.6	3.2
	Whole bark							
3	—	—	26.3	—	—	5.76	21.9	5.8
7	BWE[b]	65	23.4	59	42	12	51.3	7.8
11	H_2SO_4 2%	54	23.6	48	48	5.36	22.7	2.9
12	BWE + NaClO 2 mol/kg	64	26.7	65	35	18	67.4	11.5
13	BWE + NaClO 5 mol/kg	56	25.1	53	20	18	71.6	10.0
14	BWE + NaClO 7 mol/kg	48	25.9	47	13	21.4	83.1	10.2
15	BWE + NaOH 0.5%	55	22.3	46	nd[c]	22	98.7	12.1
16	BWE + NaOH 5%	54	28.5	58	nd[c]	22	77.3	11.9

[a]See Fig. 1 for definition
[b]BWE: boiling water extraction
[c]nd: not determined

in Table 1 (expts. 1–3) indicate that about 20 percent of the available glucose can be obtained from the four types of bark. The corresponding Y_{II} values lie between 4.16 and 6.48 g of glucose formed from 100 g of initial bark. These values are very low. In the case of spruce wood which contains about 45% potential glucose, Y_I is also of the order of 20% in the absence of any pretreatment (*3*). A sample of waste whole bark from paper mill (expt. 4) gives about the same Y_I and Y_{II} values as those obtained for native bark (expt. 3).

Reactivity of Outer, Inner, and Whole Bark Extracted with Boiling Water

The quantity of glucose formed by enzymatic hydrolysis of the preceding substrates extracted for three h with boiling water is given in Fig. 1 (curves 5–8). The limiting quantity of glucose and the yields Y_I and Y_{II} are given in Table 1 (expts. 5–8). A high increase in reactivity is observed for inner bark (expt. 6). About 58% of the potential glucose of the pretreated sample can be recuperated in this case whereas outer bark remains nonreactive. The behavior of the whole bark is, as expected, intermediate between that of its components. The loss of potential glucose as determined by quantitative saccharification is high, however. It probably corresponds to water soluble hydrolyzable tannins, starch, pectin, and a fraction of glucose containing hemicelluloses. The bark from papermill does not show any increase of reactivity after extraction with boiling water. This is difficult to explain. The long duration cold water treatment of papermill bark could have transformed the inhibitory components normally extractable with warm water into non-extractable components either by chemical reactions or by a change in the morphology of the bark. A change of the morphology of the bark could result in a decrease of the accessibility of the cellulose. The cold water treatment could also result in the development of microorganisms or in the formation of degradation products that inhibit the action of the cellulases. Y_{II} is respectively 2.2 and 14.8% for outer and inner bark.

Reactivity of Inner and Whole Bark Washed with Cold Water

It was important to compare extraction with boiling water with a less expensive treatment with cold water. This was done for inner and whole bark. Table 1 (expts. 9 and 10) shows that no increase of reactivity is observed after extraction with cold water although a rather important weight loss occurs.

Pretreatment with H_2SO_4, NaOH, and NaClO after Extraction with Boiling Water

Other pretreatments that could improve the yield of glucose were applied to bark. Extraction with diluted H_2SO_4 eliminates the hemicelluloses but results in a low yield of glucose (Table 1, expt. 11). Diluted

NaOH is well known to increase efficiently the yield of glucose obtained from straw; it degrades lignin and would eliminate tannic acids from bark. Sodium hypochlorite at pH 8 has the same properties and was shown to be particularly efficient in the case of spruce and Eucalyptus wood. Table 1 (expts. 12–17) shows that a higher fraction of the potential glucose contained in the pretreated substrate is recovered when these two pretreatments are applied to boiling water extracted bark. The yield of glucose, related to the potential glucose of the initial sample, is of the order of 40% but never exceeds 12 g of glucose/100 g of initial bark. This is quite low and economically nonvaluable.

CONCLUSION

The enzymatic hydrolysis of bark has been studied. Owing to their different anatomy and chemical composition, inner and outer bark differ in reactivity. About 20% of the potential glucose as determined by quantitative saccharification can be obtained from untreated inner, outer, and whole bark. Washing with cold water for 48 h results in the loss of 30–40% of the potential glucose. The quantity of glucose obtained from a given quantity of inner or whole bark first extracted with cold water and then hydrolyzed is still lower than that obtained from the same quantity of non-extracted bark. Extraction with boiling water for three h results in a higher loss of potential glucose (35–45%) and in a high increase of reactivity for inner and whole bark, outer bark being unchanged. The yield of glucose obtained from enzymatic hydrolysis is 50–60% of the glucose present in the extracted substrate but owing to the loss of potential glucose during the extraction, the yield reported to the initial glucose remains low. Further increase of reactivity can be obtained by reacting bark with sodium hydroxide or sodium hypochlorite which eliminate tannic acids and lignin. Although the potential glucose of the alkali treated substrate is recuperated almost quantitatively, the final yield in glucose does and could not exceed 10–12 g of glucose/100 g bark. Utilization of bark for the production of glucose thus is not valuable unless coupled with the production of fine aromatic chemicals. Study of the biodegradation of tannins and lignin is in progress.

ACKNOWLEDGMENT

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