Utilization of Waste Cellulose. IV. Comparative Study of the Reactivity of Different Substrates in the Enzymatic Hydrolysis with Trichoderma Viride

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Synopsis

Different cellulosic substrates are compared. Cellulose II (grinded Cellophane®) was demonstrated to be a very reactive substrate without any other pretreatment. Indeed, Cellophane®, even coated one side with PVDC or nitrocellulose, can be hydrolyzed to a very high yield of glucose in a reasonable time. Suspensions with an initial high content of substrate can be used. Different pretreatment (ball milling, γ-irradiation, and Fe^{2+}/H_2O_2) were found to modify the reactivity of cellulose I. The pretreatments which result in the chemical transformation of the glucose units of the initial cellulosic substrate are shown to inhibit the enzymatic hydrolysis.

INTRODUCTION

In the recent years, many studies have been concerned with the enzymatic hydrolysis of cellulose. Many papers report and discuss the rate and yields of acid and enzymatic hydrolysis as a function of the accessibility of different cellulosic substrates.[1–7] This accessibility depends on chemical and physical pretreatments of the materials. However, the lack of standardization of the experimental conditions (type of cellulase, temperature and time of hydrolysis, concentration of enzyme and substrates) makes comparisons difficult and can even lead to contradictory results. The purpose of the present work is to compare in well-defined experimental conditions the yield and rate of enzymatic hydrolysis of different types of cellulose. Several of them (coated and uncoated Cellophane® and oxidized pulp) have been seldom studied in the past.

EXPERIMENTAL

Four types of cellulosic substrates having the cellulose I crystalline structure were used. They are: cellulose A, cellulose B, newspaper cellulose, and Avicel.

The reference cellulose A is wood pulp for papermaking purpose which was characterized previously.[8] Cellulose B is dissolving pulp used for the manufacturing of Cellophane®. Nonprinted newspaper cellulose was used. Cellophane®, kindly supplied by UCB—SIDAC (Gent), has the crystalline structure of cellulose II.

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Cellulose samples (A, B, and newspaper) and Cellophane® were grinded respectively for 3 and 5 min with a type A70 apparatus from Int. Lab. App.

Ball milling (Agate balls) was performed for 12 h.

Grinded cellulose was irradiated in the dry state in the presence of air with a Gammacell 200.

For some experiments (Table II, expts 12,17,18) washing of Cellophane® was required. The following conditions were used: refluxing with H_2O for 4 h (expt 12), refluxing with tetrahydrofurane (THF) for 2 h (expt 18), and refluxing three times for 2 h with THF and hot filtration at the end of each refluxing period (expt 17).

The oxidation was realized in the following conditions: 1 g of grinded cellulose is incubated at room temperature in 500 mL of 100 m*M* acetate buffer (pH 4.2). Fe^{2+} and H_2O_2 were respectively 0.5 mM and 1 vol% in the buffer solution. In that condition, the molecular weight of the cellulose would be reduced to nearly 25% of the original value.[5] At the end of the incubation time (1, 2, or 3 days), cellulose was washed three times for 2 h with the acetate buffer and three times for 2 h with distilled water.

The characteristics of the cellulosic substrates are summarized in the Table I.

Cellulase "Onozuka R-10" from Kinki Yakult Mfg Co., Ltd., was previously characterized.[9] It is produced by a strain of Trichoderma Viride (TV).

Reducing sugars (RS) were determined by the Folin–Wu method and expressed as glucose equivalent. This method only gives an indicative value. Indeed, calibration has shown that only 50% of the cellobiose is taken into account whereas the xylose resulting from the hydrolysis of hemicellulose is counted as a 120% contribution. Furthermore, when pretreatments of cellulose involving an oxidation of the glucose units (γ-irradiation and H_2O_2/Fe^{++}) into reducing groups are used, these reducing groups could be measured if they are solubilized in the course of the hydrolysis. Glucose was analyzed by the glucose oxidase method with the "test combination glucose" supplied by Boehringer Mannheim. δ-Gluconolactone $5 \times 10^{-3}M$ was added to the tests to inhibit the β-glucosidase activity contained in the reagent.

HPLC analysis were done on a Perkin-Elmer Model 601 apparatus with an amino-bonded phase column eluted by a mixture (80/20) of acetonitrile and water.[10]

TABLE I
Molecular Weight and Crystallinity of Typical Samples

	$M_v \times 10^{-3}$ [a]	X-ray[b]	Reference
Cellulose A	135	82	8
Cellulose B	105	85	UCB–SIDAC technical data
Cellophane®	50	±60	UCB–SIDAC technical data
Cellulose ball milled	—	40	—
Cellulose A irradiated 15 Mrads	14	83	8
Cellulose A irradiated 50 Mrads	6.5	82	15

[a] M_v obtained by viscosimetry in Cadoxen solution.[15]

[b] Mean of the values obtained by Segal's method and surface method.[8]

RESULTS AND DISCUSSION

Choice of Experimental Conditions

The best experimental conditions were choosen using as cellulase complex Trichoderma Viride (Onozuka) and as substrate paper pulp cellulose A which was used as a reference in the course of this work. As with the other cellulases of TV, the higher yield of hydrolysis is obtained in buffered medium at pH 4.8. The ionic strength has only a weak influence on the hydrolysis parameters since a small inhibition on the yield of RS formed (<10%) is observed for buffer concentrations larger than 500 mM. Therefore, we chose a 100-mM citrate buffer at pH 4.8. We verified that most bivalent cations (Ca^{++}, Mn^{++}, Fe^{++}, and Cu^{++}) did not modify the course of the reaction as described in the literature.[11] Only Zn^{++} and Ca^{++} were, respectively, shown to decrease and increase the yield by a factor of 0.1 when their concentration exceeds 50 m*M*. To minimize the inhibition by the reaction products, low concentration in susbtrate (250 mg/10 mL) and in enzyme (5 FP unit/10 mL) were used. Most of the experiments reported in the literature[9] are performed between 50°C and 55°C. However, we have shown in a previous paper[9] that, in aqueous solution in the absence of substrate, the various enzymatic activities are stable at 45°C but decay rapidly at 55°C. Figure 1 shows the quantity of glucose formed as a function of the time of incubation at different temperatures. It appears clearly that for short time of hydrolysis (6 h) the optimal temperature is situated between 50°C and 55°C.

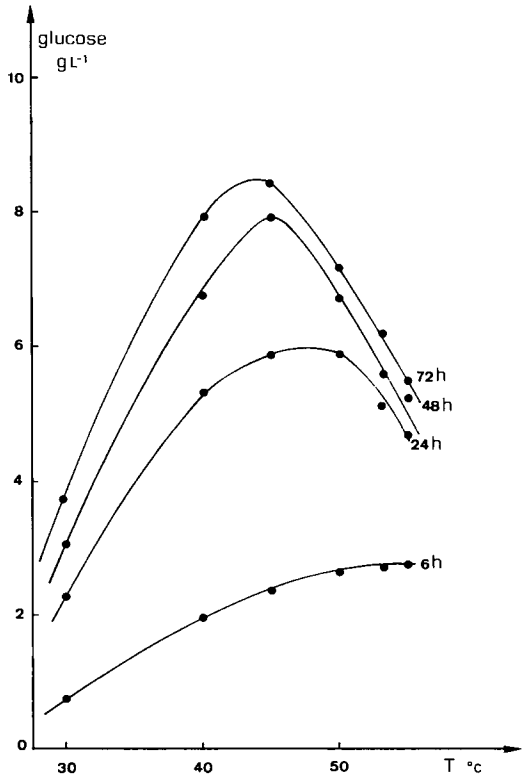

Fig. 1. Effect of the temperature on the yield of hydrolysis of cellulose A at different incubation times.

However, for longer incubation time, higher yields are obtained at a lower temperature. As a conclusion of this section, the best experimental conditions were choosen to be hydrolysis at 45°C in a 100-mM citrate buffer (pH 4.8) using a 2.5% concentration of substrate and an enzymatic activity of 0.5 FP unit/mL.

Untreated Cellulose I

Tables II and III give the quantity of glucose and total reducing sugars (glucose + cellobiose · · ·) formed for different substrates in our experimental conditions. Comparison of the data of columns 3 and 4 shows that the quantity of cellobiose formed is low. This indicates that the cellulase used has a high β-glucosidase activity in contradiction with other experiments reported in the literature[12] and performed with other enzymatic complexes obtained from TV. Cellulose A, B, and Avicel (expts 1,2,19,20,27) give similar results. Their crystalline fraction is high, although their MW are different. Newspaper cellulose (expts 3,21) is less reactive, probably because of a higher lignine content.

Cellulose II (Cellophane®)

Tables II and III (expts 1,11,20,24) and Figure 2 show that the reactivity of cellulose II (Cellophane®) is much higher than that of cellulose I (expts 1,11,20,24): in the mild hydrolysis conditions used in the present work, a yield of saccharification of more than 90% is obtained for cellulose II after 48 h whereas it is only 35% for cellulose I in the same conditions after 72 h of reaction. Cellophane® coated on one side with poly(vinylidene chloride) or nitrocellulose is

TABLE II
Glucose and Reducing Sugar Formed after 24 h Hydrolysis at 45°C

Expt	Sample	Glucose ($g \cdot L^{-1}$)	RS ($g \cdot L^{-1}$)	RS/G
1	Cellulose A (ref.)	5.4	7.3	135
2	Cellulose B	5.9	7.3	124
3	Newspaper	4.2	5.7	136
4	Cellulose A (15 Mrads)	2.45	3.5	143
5	Cellulose A (50 Mrads)	2.75	4.2	153
6	Cellulose A (100 Mrads)	3.8	6.0	158
7	Cellulose A ball-milled	9.2	13.3	144
8	Cellulose A oxydized (1 day)	4.2	4.8	114
9	Cellulose A oxydized (2 days)	3.4	3.7	109
10	Cellulose A oxydized (3 days)	3.2	3.4	107
11	Cellophane® uncoated	12.9	16.3	126
12	Cellophane® uncoated washed with H_2O	13.5	17.2	127
13[a]	Cellophane® coated with PVDC (one side)	12.1	16.0	132
14[b]	Cellophane® coated with NC (one side)	10.7	13.2	123
15	Cellophane® coated with PVDC (2 sides)	2.6	3.3	127
16	Cellophane® coated with NC (2 sides)	2.4	3.0	125
17	Cellophane® coated with PVDC (2 sides) washed with THF	10.9	13.6	125
18	Cellophane® coated with NC (2 sides) washed with THF	11.8	15.4	130
19	Avicel (microcristalline cellulose)	5.0	5.7	114

[a] PVDC = poly(vinylidene chloride).
[b] NC = nitrocellulose.

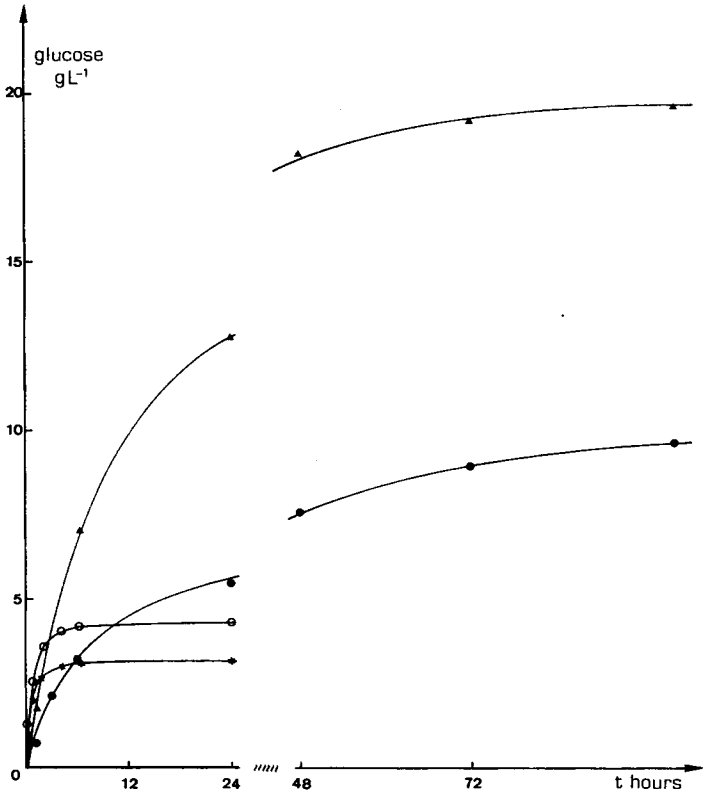

Fig. 2. Comparative yields of hydrolysis of cellulose A (●), Cellophane® (△) and carboxymethylcellulose [(○) DS = 0.55 (*) DS = 0.7] as a function of the time of incubation.

also an excellent substrate (expts 13,14,25,26). However, it is almost unreactive when it is coated on both sides (expts 15,16). The low amount of saccharification observed in this last case most probably results from enzymatic attack on the new sides formed by grinding. Indeed, an ungrinded Cellophane® film does not hydrolyze. This nonreactivity is only due to the presence of the coating since washing it out with tetrahydrofurane restores an excellent substrate for hydrolysis (expts 17,18). It must also be noted that none of the various additives present in Cellophane® deactivates the enzyme. Suspensions containing up to 20% Cellophane® can be used for hydrolysis. A typical prolonged hydrolysis is reported in Figure 3. A sirup containing about 150 $g \cdot L^{-1}$ is obtained after 11 days hydrolysis. This corresponds to about 90% of the potential RS if water, plasticizers, and other additives of Cellophane® are taken into account.

Pretreated Cellulose I

Three pretreatments presumed to increase the accessibility of cellulose and hence the yield of hydrolysis were tried: ball milling, preirradiation with γ-rays, and oxidation with Fe^{++}/H_2O_2.

Ball Milling

The ball-milling treatment used here was shown by γ-ray diffraction to reduce to 0.4 the crystalline fraction which was 0.8 in the initial sample. An important

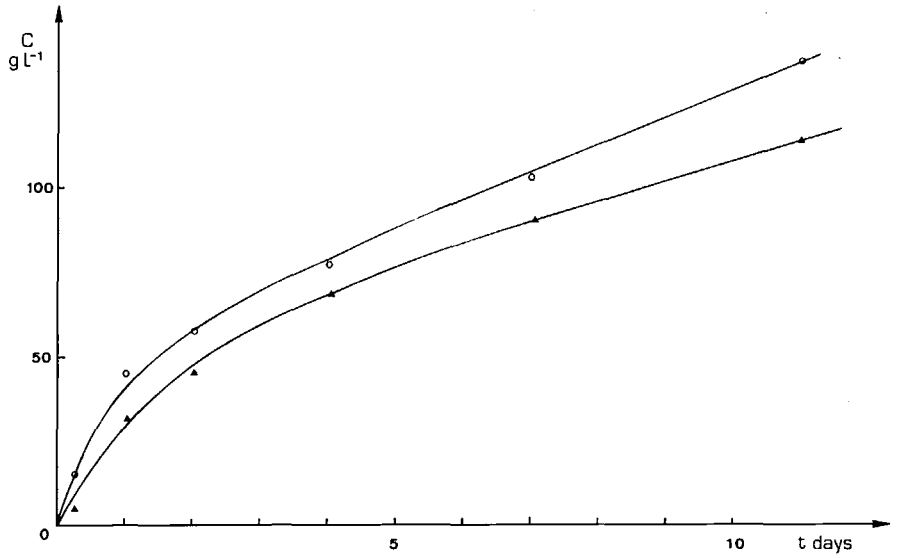

Fig. 3. Enzymatic hydrolysis of Cellophane® as a function of incubation time. Initial substrate concentration: 200 g·L^{-1}; enzymatic activity: 4 FP unit·mL^{-1}; glucose (▲); RS (○).

increase in glucose and RS formation was observed in the present work (expts 7,23) as reported previously in the literature.[1,2,4,6]

Preirradiation with γ-Rays and Oxidation with Fe^{++}/H_2O_2

The effect of γ-irradiation on the enzymatic hydrolysis is more controversial. There is less agreement in the literature concerning the effect of γ-irradiation on enzymatic and acid hydrolysis. Avella and DiGiorgio[3] report an acceleration of the enzymatic hydrolysis of cellulose after absorption of doses ranging from 1 to 200 Mrad. Kumakura and Kaetsu[13] also observed an acceleration of the enzymatic hydrolysis when working with waste lignocellulosic materials (50–500 Mrad) but a decrease of the yield of the acid hydrolysis. On the contrary, an important increase (10–20 times) of the yield of acid hydrolysis of cellulose and lignocellulosic derivatives was measured by Millett et al.[6] for an irradiation dose of 100 Mrad. A moderate increase of the yield of glucose (up to 50%) was ob-

TABLE III
Products Formed after 48-h Hydrolysis at 45°C (g·L^{-1})

Expt	Sample	Glucose, glucos-oxidase method	RS, Folin–Wu method	HP LC analysis		
				Glucose	Cellobiose	Xylose
20	Cellulose A (Ref.)	7.7	10.9	6.9	1.8	0.9
21	Newspaper	5.7	7.1	4.6	1.2	0.9
22	Cellulose A (50 Mrads)	3.8	5.7	3.5	—	0.85
23	Cellulose A (ball milling)	15.0	18.6	13.0	2.2	0.7
24	Cellophane® uncoated	18.3	20	16.0	1.2	0.3
25	Cellophane® coated with PVDC (one side)	17.3	19.2	15.6	1.4	
26	Cellophane® coated with NC (one side)	15.8	16.4	13.7	1.1	
27	Avicel (microcristalline cellulose)	7.2	8.7	6.7	0.8	

served in our laboratory for the acid hydrolysis of preirradiated (15 Mrad) paper pulp when the time of hydrolysis was shorter than that corresponding to the maximum yield in nonirradiated samples[8]; but an inhibition was observed for longer time of hydrolysis in some experimental conditions.[8] The different authors tentatively assign any observed increase in yield to a decrease of the crystallinity and of the molecular weight.

Oxidation with Fe^{++}/H_2O_2 in acid medium has been used as a pretreatment by Koenigs.[5] An increase in the yield of enzymatic hydrolysis by a factor of the order of 5 has been assigned to the important decrease of DP.

Our results (expts 4–10,22) indicate a lowering of the quantity of glucose and RS formed after 24 h in all cases of preirradiation with γ-rays or oxidation with Fe^{++}/H_2O_2. These two treatments result in a decrease of the MW as previously described, but they also transform a fraction of the glucose units. In the case of preirradiation, ketone groups are formed with a high yield. The mechanism of the radiolysis of cellulose has been discussed in a recent publication[15] as a function of new results obtained in our laboratory and other data reported in the literature. In the oxidized samples,[5] according to the experimental conditions either the C_6 carbon is oxidized to an uronic unit or the C_2 and C_3 carbons are oxidized to carboxyl groups and the pyranose ring is split. These oxidized groups could, if solubilized, react as reducing sugar by the Folin–Wu method. But Table II (expts 8,9,10) shows that pretreatment with H_2O_2/Fe^{++} does not induce any increase in the ratio RS/G. On the contrary, a decrease of RS/G (from $\pm$135 to $\pm$107) is observed. The yield of reducing sugars is weakly influenced by cellobiose since this is titrated only for 50% of its real value and is present in low and almost constant amount in all the hydrolyzates owing to the presence in the enzymatic complex of a very efficient β-glucosidase.[9] This decrease in RS/G seems thus to be due to the presence of a lower quantity of pentoses; the pretreatment by H_2O_2/Fe^{++} is indeed known to attack preferentially hemicelluloses.[5] After preirradiation, a weak increase in RS/G from 135 to 158 is observed Table II, expts 4,5,6). However, this increase is partly due to the degradation of the substrate during the irradiation. Indeed, if the irradiated substrates are suspended for 24 h at 45°C in the buffer without any enzyme, reducing sugars corresponding to 0.08, 0.17, and 0.56 g·L^{-1} of glucose equivalent are liberated. If these values are deduced from the RS values obtained after enzymatic hydrolysis, the RS/G become, respectively: 15 Mrad, 139; 50 Mrad, 146; and 100 Mrad, 143. These values are close to those obtained for the unirradiated sample. This shows that, although 15–45% of the anhydroglucose units of the cellulosic substrates have been transformed by irradiation,[6,15] very few of them have been solubilized. Carboxymethylcellulose is another example of substrate containing a fraction (70% and 55% in the present case) of transformed glucose units. Carboxymethylcellulose is soluble in water and hence very accessible to hydrolysis. Figure 2 shows that the reaction is initially very rapid in that case but limited to a few percent hydrolysis. This can be assigned to a decreased affinity of the enzyme for a transformed substrate but also to the specificity of glucanases which could hydrolyze only between nonsubstituted glucose units.[14] The carboxymethylation and oxidation of a fraction of the glucose units could thus be responsible for the low saccharification yield observed for carboxymethylcellulose, and for the irradiated and Fe^{++}/H_2O_2-treated substrates in the case of enzymatic hydrolysis. Acid hydrolysis, which is less specific, could be less sen-

sitive to chemical transformation of the glucose units and could be improved by irradiation or oxidation of the substrate.

CONCLUSION

These results show that cellulose II (Cellophane®) although highly crystalline is much more reactive than cellulose I. Moreover, the pretreatment of cellulose I in which results in the chemical transformation of the glucose units of the initial cellulosic substrate are shown to inhibit the enzymatic hydrolyss.

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