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Utilization of Waste Cellulose

VI. Pretreatment of Lignocellulosic Materials with Sodium Hypochlorite and Enzymatic Hydrolysis by *Trichoderma viride*

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ABSTRACT

A pretreatment of lignocellulosic materials with sodium hypochlorite–hypochlorous acid at controlled pH (between 7 and 9) considerably increases the accessibility of the cellulosic part of the substrate to chemical and biochemical reactants. As a consequence, the yield and rate of the enzymatic hydrolysis to glucose is largely increased. Wheat straw and spruce sawdust have been investigated. The increase in accessibility is assigned to degradation and (or) detachment of the lignin network. The loss in cellulose and hemicellulose is not important, lignin being preferentially degraded under carefully controlled pH conditions. When applied to pure cellulose, the pretreatment decreases the yield of enzymatic hydrolysis; in the absence of lignin, oxidation of the anhydroglucose units is important and results in the inhibition of the enzymatic hydrolysis.

Index Entries: Pretreatment, by sodium hypochlorite in the enzymatic hydrolysis of cellulose, wood, and straw by *Trichoderma viride* cellulases; sodium hypochlorite, pretreatment for the enzymatic hydrolysis of cellulose, wood, and straw by *Trichoderma viride* cellulases; Cellulose, wood straw, pretreatment by sodium hypochlorite for the enzymatic hydrolysis by *Trichoderma viride* cellulases; Enzymatic hydrolysis, by *Trichoderma viride* cellulases of cellulose, wood, and

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straw; lignocellulose materials, enzymatic hydrolysis of; *Trichoderma viride,* in enzymatic hydrolysis of lignocellulose materials.

1. INTRODUCTION

The cellulosic fraction of biomass is a very important and renewable source of sugars that can be converted to ethanol and other basic chemicals. The yield of glucose obtained from cellulose is, however, low becausecrystallinity limits the accessibility of the substrate to enzymatic hydrolysis. In lignocellulosic compounds, both the lignin network and the crystallinity of the cellulosic fraction are responsible for the low yield of glucose obtained. This has been recognized many years ago and a large amount of research work has been devoted to optimize the yield of enzymatic hydrolysis (*1*). This process is very specific and by consequence very attractive when compared to acid hydrolysis in which competitive degradation of the sugars formed is a nonnegligible process.

A large number of physical and chemical pretreatments have been proposed to increase the accessibility of cellulose and lignocellulosic compounds to enzymatic hydrolysis. The most important are ball milling, which reduces the crystallinity, alkali, and ammonia swelling; explosive steam decompression, which frees the cellulose fibers from lignin; and solvents such as Cadoxen that transform cellulose I into the more reactive cellulose II. All these processes have been reviewed recently (*1*). Although these pretreatments are rather performant, none of them has been developed industrially because the increase in yield is not yet sufficient to justify the cost of the operation. Pretreatment thus remains a crucial problem to be solved before there can be economical utilization of the tremendously large quantities of waste lignocellulosic materials. This problem has been investigated previously in exploratory research work performed in our laboratory (*2,3*).

The present work is concerned with the pretreatment of cellulose (paper pulp) and lignocellulosic materials (straw, spruce sawdust) with NaClO–HClO at controlled pH. The rate and maximum yield (yield after 4 d) of the enzymatic hydrolysis by *Trichoderma viride* cellulases in standard conditions are used as measurements of the accessibility of the substrate. This pretreatment has been recognized by us as very satisfactory in recent investigations (*4*).

2. Experimental

2.1. Substrates

The cellulose used is papermaking wood pulp, a form that has been described previously (*5*). The lignocellulosics are wheat straw and spruce sawdust.

2.2. Pretreatment with NaClO–HClO (3,4,6)

The pretreatment is carried out in a thermostated bath with magnetic stirring, using 0.5*M* NaClO. The same results were obtained using "pro analysi," industrial and domestic NaClO solutions. Sodium hypochlorite (0.5*M*, 50 mL) is acidified to the chosen pH value with concentrated HCl. The substrate (1.5 g) is then introduced. The pH is maintained at constant value by addition of NaOH (5*M*). After 30 min, the solid is filtered on sintered glass, then washed by stirring in water, filtered, and washed again on the filter. The pretreated substrate is then dried for 24 h at 60°C.

2.3. Weight Loss

This is measured by weighing the pretreated substrate at constant weight (24 h in aerated oven at 105°C) against the untreated one.

2.4. Quantitative Saccharification and Loss in Cellulose

The cellulosic part of the substrate (250 mg) is dissolved in 3 mL 72% H_2SO_4 (1 h at 30°C). It is then diluted with 84 mL water and kept in the oven for 2 h at 150°C. Neutralization of the acid is then performed with solid $Ba(OH)_2$. Glucose is analyzed as described in section 2.5.

2.5. Enzymatic Hydrolysis

All hydrolyses were carried out under the same conditions for comparative purposes, with the exception of those described below in section 3.5 (7). Substrate (250 mg) is soaked for 24 h at room temperature with 9 mL citrate buffer. A 10 mg sample of cellulases from *Trichoderma viride* (Onozuka R-10 from Kinki Yakult MFG Co. Ltd.) are then dispersed in 1 mL citrate buffer and added to the substrate. The hydrolysis is performed at 45°C. A volume of 10 λ is taken after convenient time intervals and analyzed for glucose by the glucosoxidase method (7) (Boehringer GOD-perid glucose). The specific activity of enzyme is 0.07 IFPU/mg.

3. RESULTS

3.1. Untreated Substrates

The quantity of glucose formed as function of time in the enzymatic hydrolysis of the untreated substrates is given in Figs. 1 and 2. The quantities formed after 4 d are, respectively, 9.6, 1.4, and 1.4 g/L for paper pulp, straw, and spruce sawdust. These data can be transformed into yields after 4 d by taking as 100% the content of potential glucose of the substrate subjected to enzymatic hydrolysis as determined by quantitative saccharification. These yields are respectively 41, 17, and 14% for paper pulp, straw, and spruce wood. Crystallinity is well known to limit

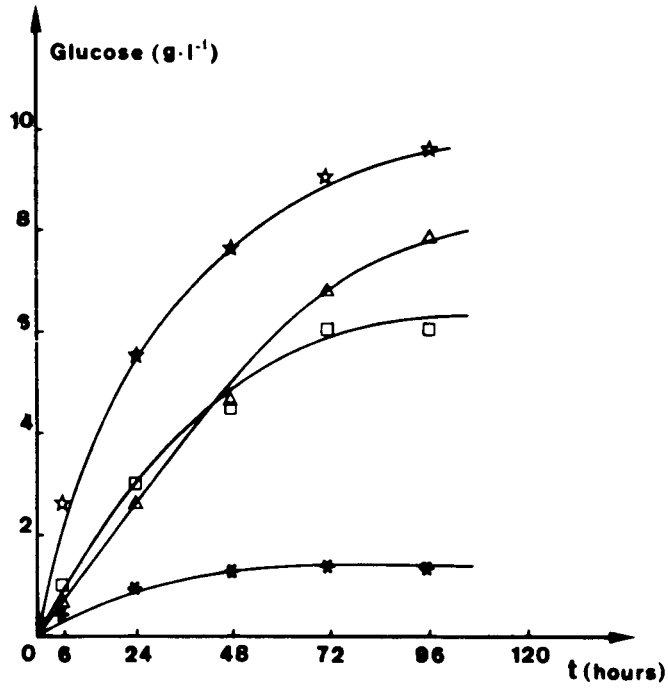

Fig. 1. Glucose formed as a function of time in the enzymatic hydrolysis of: ☆ untreated paper pulp; ★ untreated spruce; □ paper pulp pretreated with NaClO–HClO at pH 9; △ spruce pretreated with NaClO–HClO at pH 9.

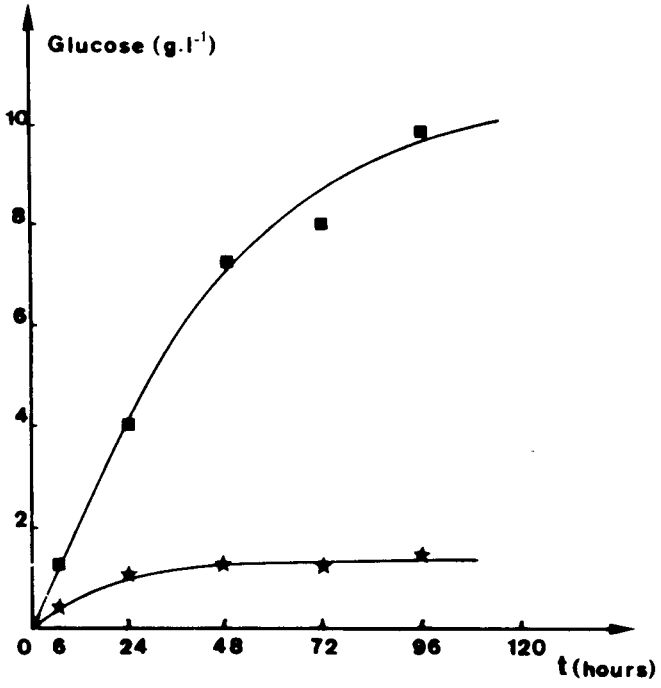

Fig. 2. Glucose formed as a function of time in the enzymatic hydrolysis of: ★ untreated straw; ■ straw pretreated with NaClO–HClO at pH 9.

the yield in glucose obtained for pure cellulose (paper pulp). In the case of untreated lignocellulosic materials, crystallinity, hemicelluloses, and lignin could be responsible for the low yields obtained even at high enzyme concentration (*3*). Hemicelluloses are, however, not involved in the limited quantity of glucose obtained. Indeed, when spruce sawdust is refluxed with dilute acid, which removes the hemicelluloses without degrading the lignin or decreasing the crystallinity, the percent conversion into glucose is still very low even at high enzyme concentration (*8*). Crystalline structure and degree of crystallinity can also be eliminated. Indeed, results obtained previously (*3*) have shown that very high yields in glucose can be obtained when lignocellulosic materials have undergone a pretreatment by HClO–NaClO, which was shown by X-ray diffraction to leave the crystallinity unchanged. Lignin is thus the most probable limiting factor. This will be further proved by the following results.

3.2. Effect of Pretreatment pH (Paper Pulp, Spruce Wood, and Straw)

The weight loss, the loss in cellulose, and the yield of glucose measured after 4 d for the enzymatic hydrolysis of cellulose have been measured for the different substrates pretreated at different pH values between 2 and 11.5.

Figure 3 shows that the weight loss is a function of the pH of the

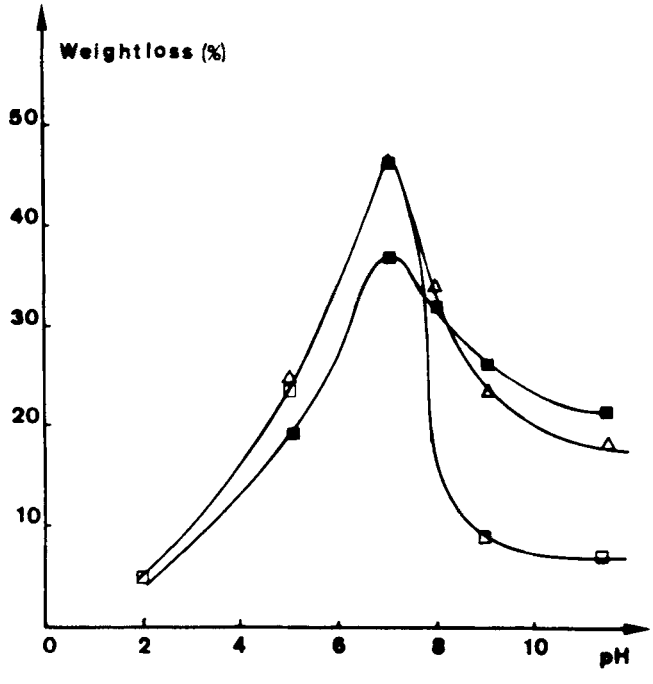

Fig. 3. Weight loss as a function of the pH of pretreatment: □ cellulose; △ spruce; ■ straw.

pretreatment. It presents a maximum in the range of pH 7–8 for all substrates.

The loss in cellulose presents a maximum in the same range of pH (Fig. 4) and is much more important for paper pulp than for the other substrates. It has been determined by quantitative saccharification of the initial and pretreated substrate followed by enzymatic analysis of the glucose formed. So, it corresponds to the difference in anhydroglucose units content of a given amount of substrate before and after pretreatment. This difference is the number of oxidized anhydroglucose units either solubilized or not.

The yield in glucose obtained after 4 d for the enzymatic hydrolysis of the lignocellulosic substrates (Fig. 5) also presents a maximum between pH 7 and 9 and is larger for straw than for spruce. Paper pulp on the contrary presents a minimum in the range of pH 5–7. With the exception of paper pulp, the initial rate and maximum yield are much higher for the pretreated substrates than for the untreated ones (Figs. 1 and 2) indicating an increase in the reactivity of lignocellulosic materials. The initial rate and maximum yield obtained after HClO–NaClO pretreatment of wood and straw are of the same order of magnitude than those obtained after a pretreatment with Cadoxen (3). They are higher than those obtained after a pretreatment with 18% NaOH (3). In the case of paper pulp, Fig. 1 reveals a decrease of the initial rate and maximum yield after pretreatment with HClO–NaClO.

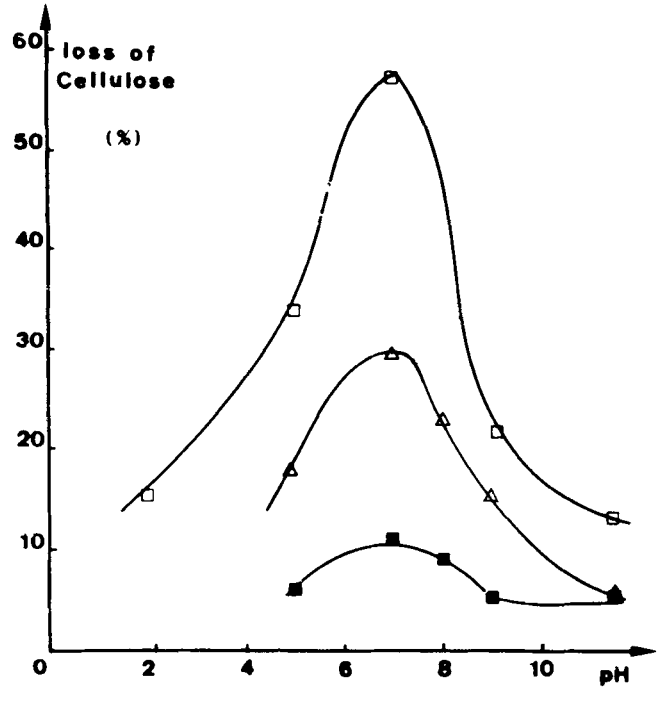

Fig. 4. Loss of cellulose as a function of the pH of the pretreatment: □ cellulose; △ spruce; ■ straw.

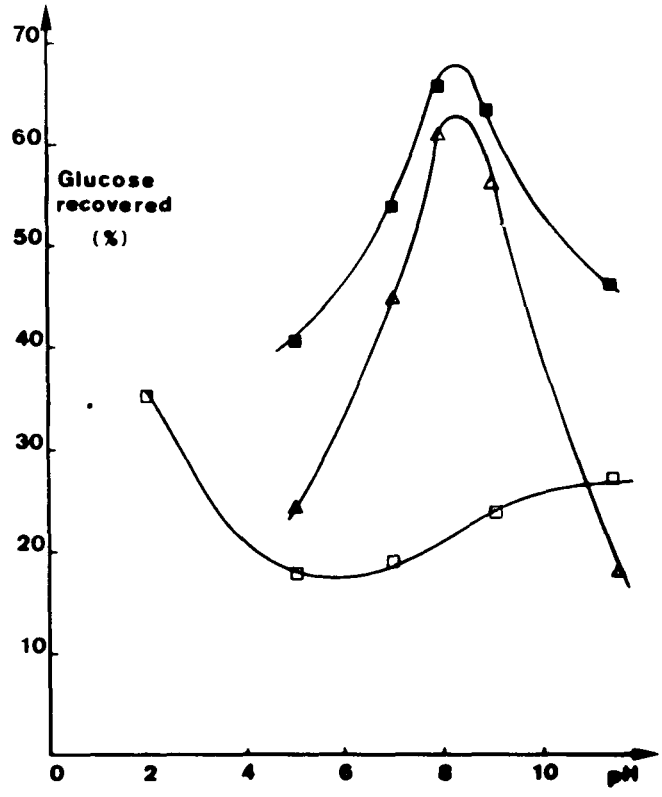

Fig. 5. Glucose formed after 4 d in the enzymatic hydrolysis as a function of the pH of the pretreatment: □ cellulose; △ spruce; ■ straw.

3.3. Effect of the Concentration of HClO–NaClO and of the Time of Pretreatment (Wheat Straw)

The results are given in Figs. 6 and 7 for straw pretreated at pH 8. These figures show that at constant time of pretreatment, the weight loss and the yield in glucose after 4 d enzymatic hydrolysis increase with increasing concentration of HClO–NaClO. The loss in cellulose is unimportant (lower than 10%) in agreement with the data of the preceding sections for straw (Fig. 4). If the time of pretreatment is varied between 10 and 30 min (standard conditions = 30 min), the weight loss and the yield in glucose first increases rapidly and then tends to a limiting value. Similar results are obtained as a function of the initial concentration of HClO–NaClO and of the time of pretreatment for pretreatments performed at pH 7 or 9.

3.4. Consumption of HClO–NaClO and NaOH (Wheat Straw)

Figures 8 and 9 give the consumption of NaClO–HClO and NaOH for straw at pH 8 as a function of the time of pretreatment and of the initial concentration of HClO–NaClO. These figures show that the ratio

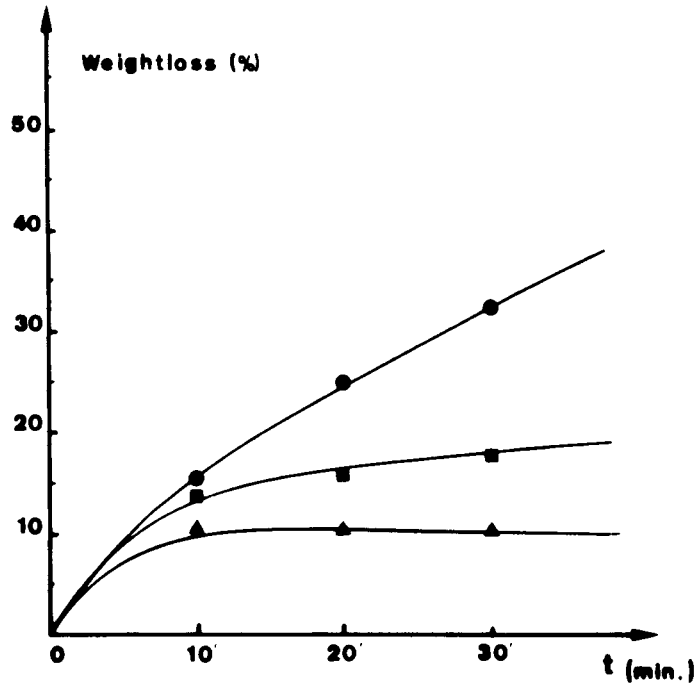

Fig. 6. Weight loss as a function of the time of pretreatment. Straw-$[HClO + NaClO]_0$: 0.5*M* (●); 0.25*M* (■); 0.1*M* (▲).

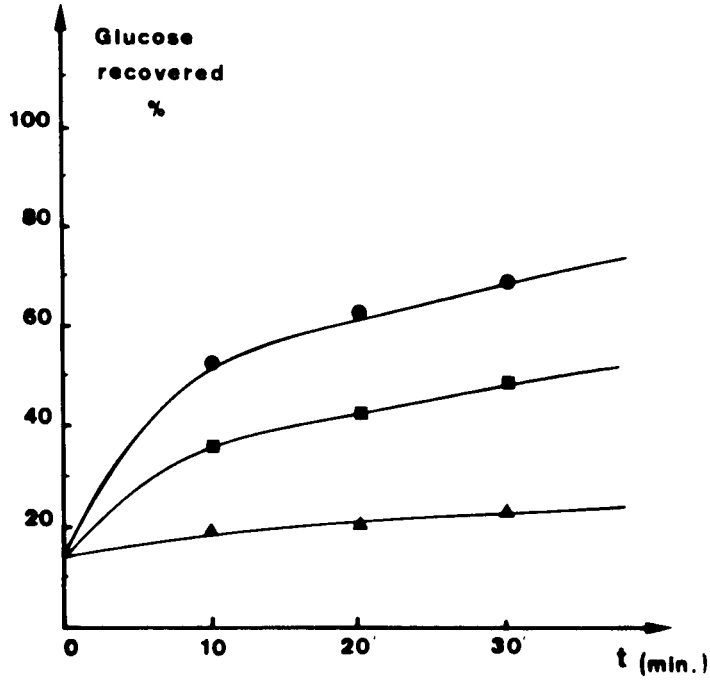

Fig. 7. Yield in glucose after 4 d as a function of the time of pretreatment. Straw-$[HClO + NaClO]_0$: 0.5*M* (●); 0.25*M* (■); 0.1*M* (▲).

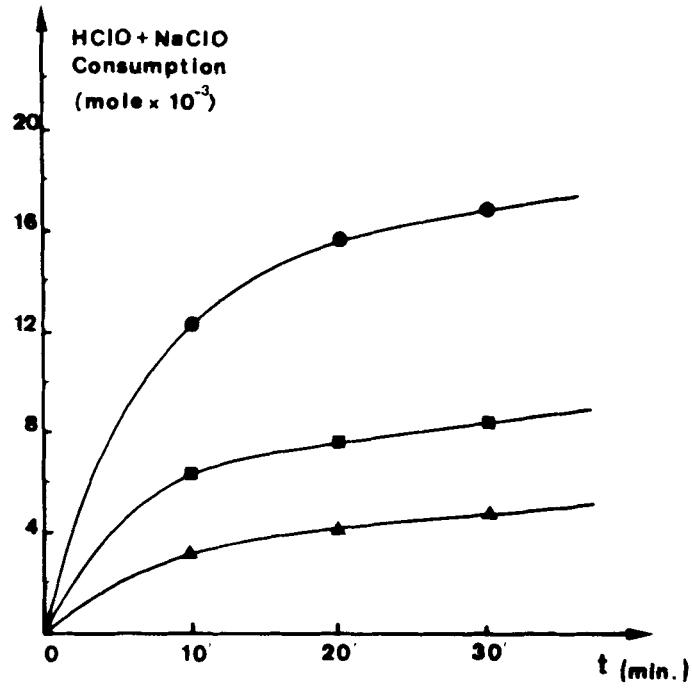

Fig. 8. HClO + NaClO consumption as a function of time of pretreatment 1.5 g Straw-$[HClO + NaClO]_0$: 0.5*M* (●); 0.25*M* (■); 0.1*M* (▲).

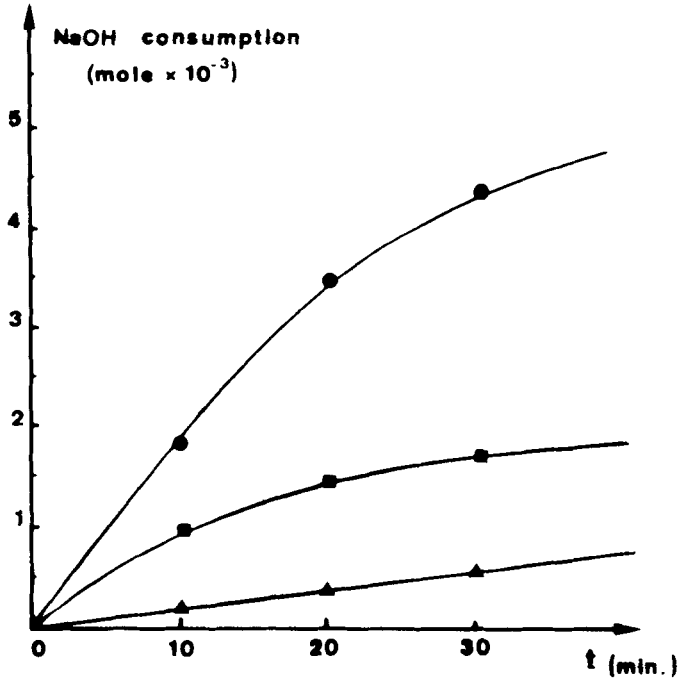

Fig. 9. NaOH consumption as a function of the time of pretreatment; 1.5 g straw $[HClO + NaClO]_0$: 0.5*M* (●); 0.25*M* (■); 0.1*M* (▲).

of HClO + NaClO to NaOH consumed varies from 4 (0.5*M*, 30 min) to 16 (0.1*M*, 10 min) when time and concentration decrease. The quantity of HClO + NaClO consumed per gram of initial dry raw straw varies from 1.27×10^{-2} to 2.34×10^{-3} mol in the same conditions. It is useful to evaluate the consumption of HClO–NaClO in mol/mol lignin units contained in the untreated substrate. Indeed, raw dry straw contains about 15% lignin, 25% hemicellulose, 35% cellulose, and 10% extractives. It can reasonably be assumed that the weight loss is mainly that from the oxidation of lignin and loss of extractives; indeed, careful analysis of Eucalyptus Saligna meal pretreated in the same conditions (*8*) has shown that loss of hemicelluloses is very low (<20% of the initial hemicelluloses content). With these assumptions, about 10 mol of HClO–NaClO are consumed per mole of lignin units for a 10 min pretreatment at pH 8 when $[HClO + NaClO]_0$ is 0.5*M*. Values of this order of magnitude are reported in the literature for hypochlorite oxidation of different types of lignin and model compounds (unmethylated). The consumption of HClO + NaClO and of NaOH as a function of pH is given in Fig. 10 for a 30 min pretreatment when $[HClO + NaClO]_0 = 0.5M$. When the pH increases, oxidation is less important, as revealed by decreasing consumption of HClO + NaClO. Furthermore, the proportion of acids in the oxidation products decreases with increasing pH of pretreatment, as indicated by the increase of [HClO + NaClO]/[NaOH] consumed.

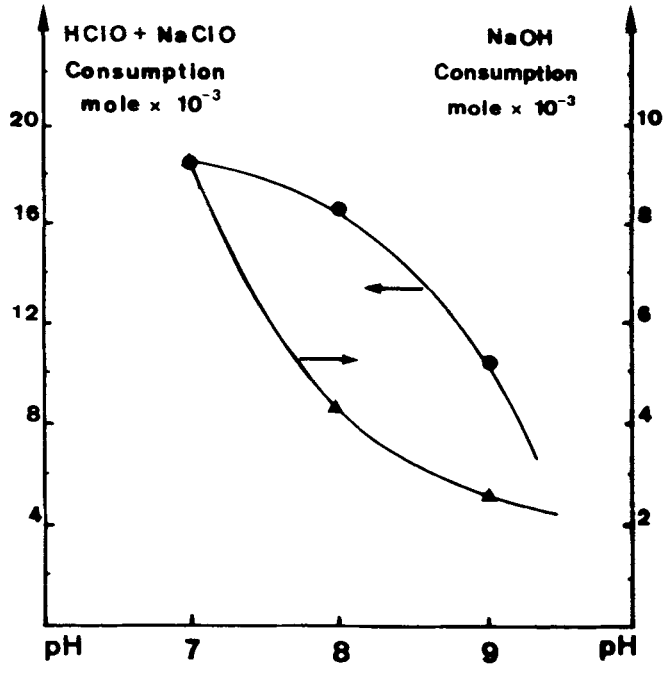

Fig. 10. HClO + NaClO and NaOH consumption as a function of the pH of pretreatment; 1.5 g straw-$[HClO + NaClO]_0$: 0.5*M*.

3.5. Effect of the Temperature of Pretreatment (Wheat Straw)

The experimental results are given in Fig. 11. They are not obtained at constant pH, as in the preceding sections. The initial pH is about 11.5 and it changes as a function of time. These experiments are another proof that a pH value of 7–9 has to be maintained for a sufficient time interval to obtain an efficient pretreatment. The final pH values after 15 min are, respectively, 11.5 and 11 for 20 and 30°C. At these temperatures, the loss in weight and the loss in cellulose are low (<10%) in agreement with the results obtained at a constant pH value of 11.5 reported in the preceding sections for straw. At 40 and 50°C, the pH spontaneously decreases to 9 and 8 owing to the formation of acid groups by oxidation of cellulose and lignin. In agreement with the experiments performed at a constant pH value of 8 and 9, the yield in glucose is higher than that obtained at 20 and 30°C, but the loss of cellulose during the pretreatment is rather important (Fig. 11). As a consequence, pretreatments with free pH evolution at 40–50°C are not favorable.

4. DISCUSSION

The experimental results show that the reactivity of lignocellulosic materials in enzymatic hydrolysis is largely enhanced by a pretreatment with HClO–NaClO, while the reactivity of pure cellulose decreases.

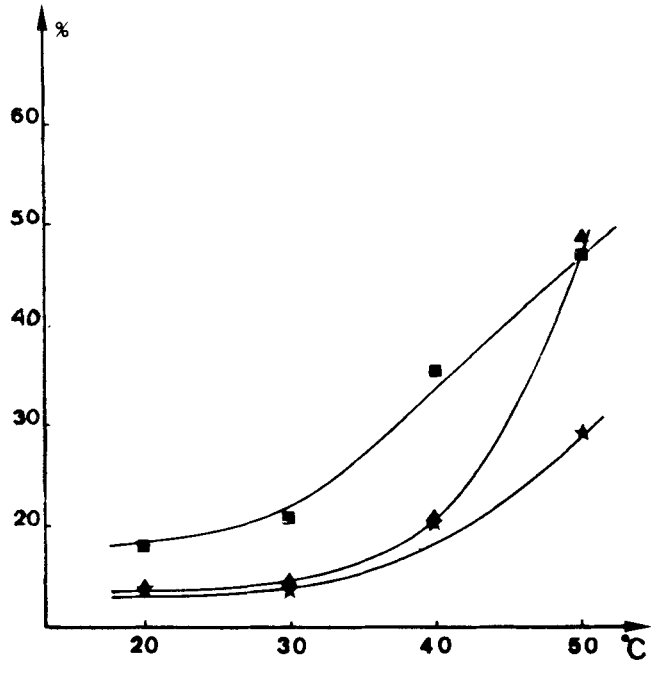

Fig. 11. Yield in glucose after 4 d (■), loss of cellulose (★), and weight loss (▲) as a function of the temperature of pretreatment-straw.

The decrease in reactivity of pure cellulose is maximum when the pH of the pretreatment lies in the range 5–7. To this loss in reactivity is associated a loss in cellulose caused by oxidation of the anhydroglucose units of the substrate. The oxidation of cellulose by NaClO is well documented in the literature. Acid, aldehyde, and ketone groups are formed. The effect of the pH on the rate of formation of carboxyl and of aldehyde groups on cellulose has been reported (*9*). They were measured, respectively, by methylene blue absorption and by the copper number. The results given in Table 1 show that the rate of degradation is the highest at pH 7. The nature of the oxidized groups formed on cellulose has been investigated more recently by Lewin and Epstein at constant oxygen consumption (*10*). Their results, also given in Table 1, show that acid groups are preferentially formed at high pH whereas reducing groups are more readily formed at low pH. The loss of cellulose and the decrease in reactivity observed in the present work by enzymatic methods are thus caused by the introduction of foreign functional groups on the cellulose backbone. Cellulases and glucosoxidases are indeed highly selective reactants that do not recognize oxidized anhydroglucose units. In addition to the formation of oxidized groups on the cellulose, an important part of the substrate is degraded to low molecular weight soluble products, as indicated by the loss in weight.

In the case of lignocellulosic materials, cellulose, hemicelluloses, lignin, and extractive products could all be oxidized. Acid, aldehyde, and ketone groups can be formed on hemicelluloses as on cellulose. Figure 4, however, shows that the loss of cellulose is much lower for straw than it is for paper pulp in the same conditions. From this, it can be deduced and has been proved on other lignocellulosic substrates (*8*) that lignin is more degradable than cellulose and hemicelluloses. The main parameter responsible for the increase in accessibility seems thus to be the reaction of NaClO–HClO with lignin. The lignin network is partly degraded into soluble products (*11*) and partly detached from the cellulose fibers by breaking the lignin–carbohydrate bonds. This detached lignin probably remains trapped in the fiber walls protecting cellulose against NaClO attack, but allowing accessibility to enzymes. Oxidation of lignin by NaClO is proposed by Sarkanen (*11*) to result from an initial chlorination of the phenolate ions by HClO, followed by oxidation with ClO^-. The proportion of HClO and free phenolate ions present in the medium are given in Table 1. Examination of this table shows that pH 7–9 corresponds to intermediate proportion of HClO and ϕO^- and could justify the maximum yield in glucose and delignification observed in this range of pH.

The increase in reactivity of the lignocellulosic materials could also result from a transformation of cellulose I into cellulose II by the pretreatment. However, it has been shown recently by X-ray diffraction that formation of cellulose II is unimportant in these conditions (*3*).

Different criticisms can be advanced against the pretreatment by HClO–NaClO at pH 8. They are: the limited yield in glucose reported in

TABLE 1
Oxidation of Cellulose by HClO–NaClO as a Function of pH

pH	ϕ/O^{-}[a] / ϕ/OH	HClO[b] / HClO + ClO^{-}	Rate of formation of acid groups (relative units), g	Rate of formation of reducing groups (relative units), g	Functional groups, mmol/100 g cotton oxygen consumption: ±130 mEq/100 g (10) COOH	CHO	CO
5	3×10^{-6}	1	1.5	0	3.03	9.87	13.2
7	3×10^{-4}	0.77	18	16	3.53	9.0	9.6
8	3×10^{-3}	0.25	3	1	5.84	8.0	7.0
9	3×10^{-2}	0.03	1	0.5	9.10	5.1	1.7
11.5	10	$\approx10^{-4}$			13.40[c]	0.34[c]	0.0[c]

[a] pK_a, substituted phenols ≈10.5; ϕO^{-} = substituted phenolate ion; ϕOH = substituted phenol.
[b] pK_a, HClO = 7.5.
[c] pH = 10.

the present work (61% of the theoretical value), the loss of cellulose, the use of energy-intensive sawdust, the loss of the lignin, and the formation of contaminant salts. A detailed comparative kinetic study of the enzymatic hydrolysis of pretreated and untreated spruce wood sawdust has shown (*12*) that the initial rate and limiting yield increase with the initial quantity of enzyme. Yields over 90% of the theoretical value are obtained for the pretreated substrate using a twofold increase either in enzyme concentration or in the time of hydrolysis. Eucalyptus wood is particularly interesting to transform into glucose because it is a very rapidly growing species. Recent results (*8*) have shown that in this case, the loss of cellulose during the pretreatment is very low (<10%) and that the properties of pretreated chips are comparable to those of pretreated meal (*12*). The loss of the lignin part of wood and the formation of contaminant salts could not be obviated until now.

In spite of these last drawbacks, the initial rate of hydrolysis and the yield of glucose formed in 4 d after pretreatment with HClO–NaClO at pH 8 compares very favorably with other chemical pretreatments when hydrolysis is performed in standard comparative conditions (*3*). Indeed the yield is 61% for the present pretreatment and, respectively, 41 and 62% for 18% NaOH (2 h) and Cadoxen (24 h).

5. CONCLUSION

Pretreatment of lignocellulosic materials with NaClO increases very effectively its accessibility to enzymes when performed near pH 8. However, it may not have been applied to pure cellulose waste if enzymatic reactions must be performed on the pretreated substrate since its reactivity is decreased as a result of chemical modifications.

Optimization of the other conditions (time of pretreatment, temperature, ratio of NaClO to substrate) needs, however, to be carried out for every material and for every planned application, taking the economic point of view into consideration. Indeed, the loss in weight and the loss in cellulose can be different for different lignocellulosic materials under the same conditions because of their particular morphologies. In any case, pretreatments of half an hour at room temperature are good, if not the best, conditions. The increase in accessibility results mainly from competitive detachment and degradation of the lignin network and oxidation of the anhydroglucose units.

ACKNOWLEDGMENT

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