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Comparison of Steam Pretreatment of Eucalyptus, Aspen, and Spruce Wood Chips and Their Enzymatic Hydrolysis

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ABSTRACT

Enzymatic hydrolysis of SO_2-impregnated, steam-exploded *Eucalyptus viminalis* was carried out at increasing substrate concentrations and enzyme loadings. When low enzyme loadings were used, the peroxide-treated fraction derived from eucalyptus chips (SEE-WIA/H_2O_2) was more readily hydrolyzed than the water-insoluble (SEE-WI) and the alkali-insoluble (SEE-WIA) fractions. The various cellulosic fractions derived from steam-exploded *E. viminalis* exhibited a greater susceptibility to hydrolysis than the equivalent aspen and spruce substrates, particularly at high substrate concentrations (10%, w/v).

Index Entries: Steam pretreatment; lignocellulosic residues; enzymatic hydrolysis; *Eucalyptus viminalis;* hardwoods/softwoods.

INTRODUCTION

The utilization of lignocellulosic residues as a potential low-cost carbohydrate source for glucose and ethanol production has been hindered by the low efficiency by which the cellulosic portion of these materials is enzymatically hydrolyzed to monomeric sugars. Several types of pretreatment have been shown to be effective in enhancing the enzymatic hydrolysis of wood residues (*1–6*). Acid-catalyzed steam pretreatment appears to be one of the most cost-effective methods for providing effective fractionation of the three major wood components and ensuring effective hydrolysis of the cellulose component in a variety of lignocellulosic

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residues (*2,3,7–12*). Recent work has shown that acid catalysis, using low levels of sulfur dioxide (SO_2) addition to the wood chips, significantly enhanced the enzymatic saccharification of steam-treated substrates derived from a variety of hardwoods and softwoods (*13,14,15*). Several other advantages have also been associated with the use of SO_2 catalysis. These include the easy and even incorporation of the acid catalyst within the wood chips, a substantial reduction in time and temperature requirements for the steam treatment, an enhanced fractionation and recovery of wood components, and an increased survival of hemicellulose-derived sugars.

Although the mode of action of SO_2 catalysis during the steam pretreatment has not yet been completely resolved, it appears that the SO_2 gas is partially incorporated within the wood chips as sulfurous acid and then subsequently converted into sulfuric acid during steam pretreatment (*16*). Recent findings have shown that this process is facilitated when wood chips with a relatively high (100%, w/w) moisture content are used, i.e., "green" conditions (*14*). This previous work only investigated the steam pretreatment of SO_2-impregnated wood chips in the absence of explosive decompression.

Although several factors have been suggested to explain why cellulosic substrates are not completely hydrolyzed at high substrate concentrations, the redistribution of lignin within the substrate during the substrate preparation (*13,15*) and the structural modifications of the cellulosic substrate during the course of hydrolysis (*7*) are often cited as the major substrate-related factors. Enzyme-related factors may also contribute to incomplete hydrolysis. These probably include the accumulation of sugars during hydrolysis leading to end-product inhibition (*17*), the irreversible and/or nonspecific adsorption of cellulases onto the substrate (*18*), and inactivation of key components of the cellulase complex.

Previous results have indicated that the enhanced accessibility of the cellulose component of steam-treated substrates to the enzymes appears to be related to the ability to extract the hemicellulose and lignin components after pretreatment. The extraction of hemicellulose and lignin causes a substantial increase in the surface area of the cellulose available to the enzyme, thus increasing substrate hydrolysis.

The influence of other structural factors, such as cellulose crystallinity and degree of polymerization on the ease of hydrolysis of the pretreated substrate, has not yet been completely resolved. We have shown that the steam pretreatment results in a slight increase in the crystallinity index of the substrate, whereas the degree of polymerization of its cellulose component decreases with increasing severity of the pretreatment.

Much of the past work on enzymatic hydrolysis of cellulose has utilized high enzyme loadings in order to minimize the negative effects of end-product inhibition (*11*). Alternatively, other authors utilized very low substrate concentrations to evaluate the susceptibility of the substrate to hydrolysis (*9*). In this work, we have studied the enzymatic hydrolysis

profile of steam-pretreated *Eucalyptus viminalis* derived from 6–7 yr-old stems, and compared the hydrolysis profile with those obtained previously with steam-pretreated spruce (*Picea albis*) and aspen wood (*Populus tremuloides*) (*13,15*).

MATERIALS AND METHODS

Steam Pretreatment

The steam pretreatment was performed in a steam gun as described previously (*19*). SO_2-impregnated chips were treated by steam for various times and temperatures using the conditions described previously (*13,14,15*). SO_2-impregnated steam-exploded substrates derived from eucalyptus, aspen, and spruce wood chips were termed SEE, SEA, and SES, respectively. The pretreatment conditions used for each wood species are shown in Table 1. The resulting steam-treated substrates were extracted twice with water at a 5% (w/v) solids concentration at room temperature (RT) and the respective water-insoluble fractions (WI) were termed SEE-WI, SEA-WI, and SES-WI. These water-insoluble fractions (WI) were subsequently extracted with alkali (NaOH 0.4% [w/v]) at 5% (w/v) solids concentration at RT, and the resulting alkali-washed substrates (WIA) were termed SEE-WIA, SEA-WIA, and SES-WIA. Alkaline peroxide treatment (*4*) of the WIA fractions was carried out at 2% (w/v) solids concentration using 1% (w/v) H_2O_2, pH 11.5, at RT for 12 h. The respective peroxide-treated substrates were termed SEE-WIA/H_2O_2, SEA-WIA/H_2O_2, and SES-WIA/H_2O_2.

Substrate Analysis

Lignin content was determined by TAPPI Standard Method T222 os-74 for acid-insoluble lignin (Klason lignin). The substrate was hydrolyzed to its component sugars during Klason lignin determination, and total carbohydrates were determined by HPLC, using an HPX-87H column (Bio-Rad) for acidic samples and an HPX-87P column (Bio-Rad) for neutralized samples (*20*). Acid-soluble lignin was determined by TAPPI Useful Method 250.

Enzymatic hydrolyses were performed at various substrate concentrations using Novo Celluclast cellulase preparation (Novo, Denmark), which was supplemented with Novozym β-glucosidase preparation to give an enzyme mixture with a final filter paper to β-glucosidase activity ratio of approx. 1:3, respectively (*21*). Enzymatic activities were determined in the enzyme mixture as previously described (*22,23*). Hydrolyses were carried out in a 0.1*M* sodium acetate buffer solution at 45°C and 145 rpm using tetracyclin (0.0006% [w/v]) as a preservative. Hydrolysis yields were measured using HPLC as described previously (*22*). The enzyme

Table 1
Pretreatment Conditions for Enhanced Enzymatic Hydrolysis and Recovery Yield of Steam-Treated Substrates Derived from Eucalyptus (SEE), Aspen (SEA), and Spruce (SES) Wood Chips

	Treatment Conditions			Recovery Yield[2]		Klason Analysis	
Substrate[1]	Temperature, °C	Time, s	$[SO_2]$, g/100 g	Substrate, g	Cellulose, g	Cellulose,[3] g/100 g	Lignin, g/100 g
Eucalyptus viminalis							
Untreated	-	-	-	100	100	41.4	27.0
SO_2-SEE-WI	210	50	1.0	64.6	91.4	58.6	30.4
-WIA				45.2	92.1	84.4	5.4
-WIA/H_2O_2				41.8	91.0	90.1	0.6
Populus tremuloides (Aspen)[4]							
Untreated	-	-	-	100	100	42.7	20.9
SO_2-SEA-WI	210	100	1.6	56.9	83.4	62.6	30.3
-WIA				35.3	71.0	85.9	5.4
-WIA/H_2O_2				33.0	66.9	86.6	2.2
Picea albis (Spruce)[5]							
Untreated	-	-	-	100	100	40.4	27.3
SO_2-SES-WI	210	150	2.5	60.8	75.6	50.2	45.8
-WIA				51.2	74.4	58.7	37.1
-WIA/H_2O_2				37.8	75.0	80.2	15.0

[1]SEE, steam-exploded eucalyptus; SEA, steam-exploded aspen; SES, steam-exploded spruce wood; WI, water-insoluble fraction; WIA, alkali-insoluble fraction; WIA/H_2O_2, peroxide-treated fraction.
[2]Expressed in terms of oven-dry wood weight.
[3]Determined by HPLC analysis of the substrate hydrolysate obtained during Klason lignin determination.
[4]Extracted from Schwald et al. (*13*) and Breuil et al. (*21*).
[5]Extracted from Schwald et al. (*15*) and Breuil et al. (*21*).

loadings used in this study were calculated based on the actual cellulose content of the pretreated substrates. Enzyme loadings and hydrolysis yields can be calculated in terms of the cellulose content of the original, untreated wood chips by considering the cellulose recovery yields given in Table 1 for the different pretreatment conditions.

RESULTS

In earlier work with eucalyptus (*14*), aspen (*13*), and spruce (*15*), we determined the best steam treatment conditions for enhanced enzymatic hydrolysis and recovery yield of the pretreated fractions. We generally found that the more drastic the pretreatment, the lower the recovery yield and the better the enzymatic accessibility of the resulting substrate (*2*).

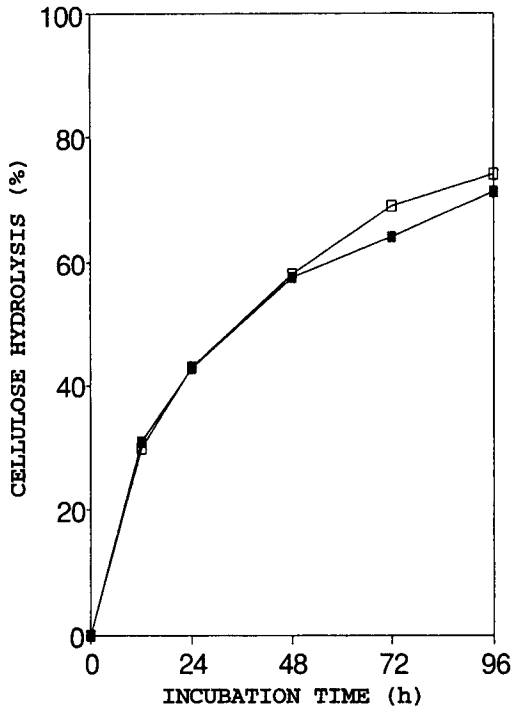

Fig. 1. Enzymatic hydrolysis profile of the (■) SEE-WI and (□) SEE-WIA fractions carried out at 5% (w/v) cellulose concentration using 10 FPU g^{-1} of cellulose.

The conditions summarized in Table 1 are those in which the best substrate for hydrolysis was produced with the least material lost because of decomposition reactions (*13,14,15*). It was apparent that the steam treatment of eucalyptus chips resulted in slightly better recovery yields, as compared to aspen and spruce chips. Subsequent water and alkali extraction of the steam-exploded substrates was shown to be beneficial, since it separated the majority of the hemicellulose and lignin components into different fractions for further processing. We also hoped that the removal of most of the hemicellulose and lignin would increase the accessibility of the cellulose to the cellulase enzymes. Although alkali extraction could remove a substantial amount of the original lignin present in aspen and spruce wood, hydrolysis was only marginally increased for the former substrate and was significantly reduced for the latter (*13,15*). It has been suggested that the redistribution of lignin or an alteration in the crystalline structure of the cellulose could be responsible for the reduced degree of hydrolysis of alkali-washed substrates (*13,24*).

We next compared the hydrolysis profiles obtained when the water-extracted (SEE-WI) and the alkali-extracted (SEE-WIA) fractions of steam-exploded eucalyptus had 10 FPU g^{-1} cellulose added to each substrate at a 5% (w/v) cellulose concentration (Fig. 1). It was apparent that the

Table 2
Effect of Increasing Enzyme Loadings
on the Enzymatic Hydrolysis of the Alkali-Insoluble Fraction
Derived from Steam-Exploded Eucalyptus Wood Chips (SEE-WIA)

Substrate loading[1]	Enzyme loading, FPU/g[2,4]	Glucose yield, %[3,4] Incubation time, h					
		06	12	24	48	72	96
2% (w/v)	3.1	14.4	21.4	28.0	44.6	57.7	-
	5.1	20.7	33.3	44.2	68.3	80.7	-
	8.7	28.3	43.4	60.5	85.2	96.8	-
	15.1	41.3	-	82.7	101.4	103.2	-
	19.7	47.0	-	90.9	100.1	103.6	-
10% (w/v)	9.9	18.8	28.1	39.7	52.6	60.0	68.6
	10.3	-	28.5	40.8	54.2	65.2	71.1
	17.4	-	32.4	44.2	61.3	75.8	82.1
	20.7	-	36.0	50.7	69.0	81.1	84.9

[1]Expressed in terms of oven-dry weight.
[2]Amount of enzyme added to the reaction mixture, expressed as FPU g^{-1} cellulose.
[3]Determined in the hydrolysates by HPLC and expressed as percent of the theoretical glucose yield attainable from the initial pretreated substrate.
[4]Enzyme loadings and hydrolysis yields can be expressed in terms of the original cellulose content of the untreated wood chips by multiplying the values by 0.921 (Table 1).

hydrolysis profiles were similar and that the lignin present in SEE-WI did not significantly restrict hydrolysis. However, neither substrate was completely hydrolyzed after 96 h of incubation. When these recalcitrant residues were examined by scanning electron microscopy (SEM) (data not shown), the cellulose fibers and parenchyma cells were found to be attacked to a much greater extent than tracheary elements, such as the plant vessels. Fiber bundles also accumulated in the recalcitrant residue, and they may represent the proportion of the original wood chips that were incompletely steam-treated.

Hydrolyses of the pretreated substrates were routinely carried out using Novo Celluclast supplemented with an excess of β-glucosidase (Novozym). This was to ensure that we could achieve maximum hydrolysis by restricting the accumulation of cellobiose, which could lead to end-product inhibition (*21*). Ideally, we wanted to provide a substrate that could be hydrolyzed rapidly and completely to glucose with as little enzyme as possible. We therefore compared the extent of cellulose hydrolysis when increasing concentrations of enzyme were added to 2 and 10% (w/v) concentrations of the alkali-extracted substrate (SEE-WIA) (Table 2). At a 2% (w/v) concentration, all of the cellulose could be hydrolyzed after a 48-h incubation, when an enzyme loading of 15 FPU g^{-1} cellulose was used. Even at an enzyme loading of 8.7 FPU g^{-1} cellulose, almost 97% of

the original cellulose could be detected as glucose after a 72-h incubation. In some cases, values greater than 100% were obtained. This probably reflects a problem in quantifying the amount of cellulose originally present in the substrate. Higher enzyme loadings seemed to be more important when the higher substrate concentration was used. Only 85% of the original cellulose was hydrolyzed to glucose after 96 h of incubation at an enzyme loading of 20.7 FPU g^{-1}. There was no linear relationship between the efficiency of hydrolysis and the amount of enzyme added to the substrate.

Hydrolysis profiles for the alkali-extracted (SEE-WIA) and peroxide-treated (SEE-WIA/H_2O_2) fractions were obtained at 2 and 10% (w/v) substrate concentrations using 10 FPU of enzyme/g of cellulose (Fig. 2). Although the peroxide treatment increased the efficiency of hydrolysis at both substrate concentrations, it did not appear to result in as dramatic an increased as had been obtained previously with aspen or spruce wood (*13,15*). We next carried out a direct comparison between the three substrates that had been pretreated using the conditions described in Table 1 (Fig. 3). At a 2% (w/v) substrate concentration, both aspen (SEA-WIA/H_2O_2) and eucalyptus (SEE-WIA/H_2O_2) showed a similar hydrolysis profile. Although spruce (SES-WIA/H_2O_2) exhibited a slower rate of hydrolysis, it could still be completely hydrolyzed after 96 h. At higher (10%, w/v) substrate concentrations, eucalyptus demonstrated substantially better hydrolysis, whereas aspen hydrolysis fell between eucalyptus and spruce.

DISCUSSION

Previous work with *E. viminalis* has shown that the steam treatment of SO_2-impregnated chips produced a substrate that could be effectively fractionated with the cellulose component made highly accessible to cellulolytic enzymes (*14*). Although it appeared that the alkali-washed substrate derived from *E. viminalis* exhibited a lower accessibility than the water-insoluble fraction, this was probably because of the higher cellulose content of the former resulting in a higher degree of end-product inhibition of the enzymes. It was apparent that alkali washing did not interfere with the accessibility of steam-exploded *E. viminalis* even when high substrate concentrations of 5% (w/v) cellulose were used. Similar results were obtained when steam-treated substrates derived from other hardwood species, such as aspen (*13*), were utilized. Although the greater accessibility of the peroxide-treated fraction derived from *E. viminalis* (SEE-WIA/H_2O_2) was partially owing to its very low lignin content (Table 1), other structural factors, such as the fibrilation of the cellulose fibers and a reduction in the degree of polymerization of cellulose, probably led to better "swelling" of the substrate.

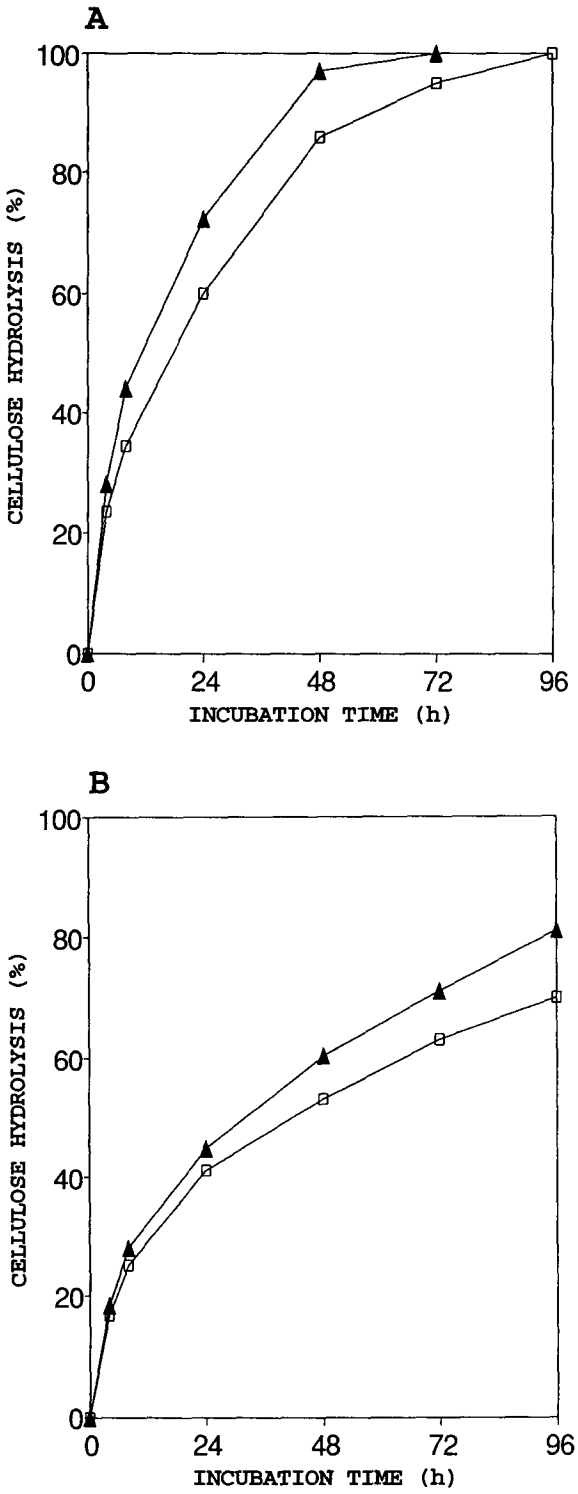

Fig. 2. Enzymatic hydrolysis profiles of steam-treated substrates derived from eucalyptus wood chips. Hydrolyses were carried out at (A) 2% and (B) 10% (w/v) substrate concentrations using 10 FPU g^{-1} cellulose. (□) SEE-WIA, alkali-insoluble fraction; (•) SEE-WIA/H_2O_2, peroxide-treated fraction.

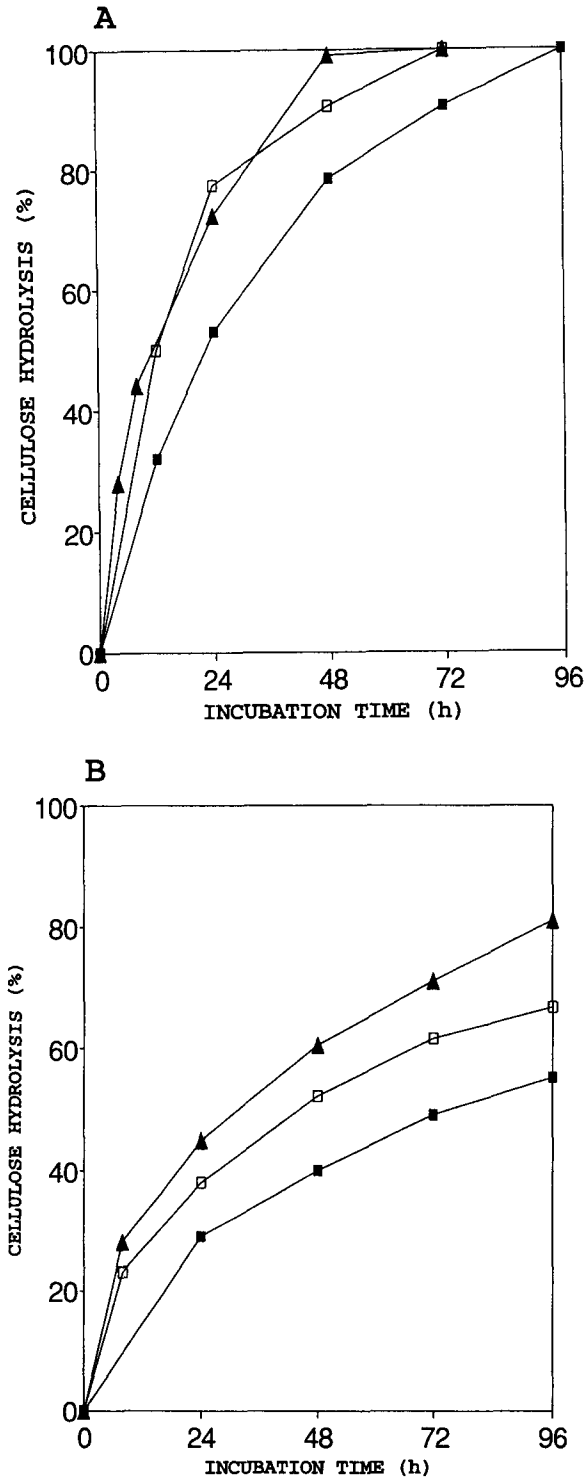

Fig. 3. Comparison of the hydrolysis profile of the peroxide-treated fractions derived from steam-treated (■) *E. viminalis* (SEE-WIA/H_2O_2), (□) aspen (SEA-WIA/H_2O_2), and (▲) spruce (SES-WIA/H_2O_2) wood chips. Hydrolyses were carried out at (A) 2% and (B) 10% (w/v) substrate concentrations using 10 FPU g^{-1} cellulose.

Large vessel elements are the most accessible hardwood cell type, yet they appear to be extremely resistant to enzymatic attack. SEM examination of the ultrastructure of decayed stumps of red alder indicated that libriform fibers and ray parenchyma cells were almost totally degraded, whereas vessel elements remained relatively unmodified after extensive deterioration of the stem wood (*25,26*). It appeared that high levels of deterioration were only observed where syringyl lignins were predominant and that the lower accessibility of plant vessels was partially the result of the occurrence of a more recalcitrant guaiacyl lignin type in the vessel walls. A selective degradation of libriform fibers and parenchyma cells was also observed during the enzymatic hydrolysis of acid-pretreated substrates derived from birch wood (*27*).

Previously, it had been shown that lignin extraction with alkali decreased the rate and extent of hydrolysis of steam-treated substrates derived from softwood species, such as spruce (*15*) and *Pinus radiata* (*28*). It appeared that it was primarily the nature and the redistribution of the guaiacyl lignins found mostly in softwoods that restricted the hydrolysis of the cellulose from steam-treated spruce. When the residual substrate remaining after extensive hydrolysis of steam-treated aspen and eucalyptus was examined microscopically, it was apparent that this debris was mainly composed of vessel elements. Since the lignin in vessel elements is known to have a greater guaiacyl to syringyl ratio than other cells found in hardwood tissues, it is probable that it is the distribution of this lignin fraction that restricts the swelling of the cellulosic residue and reduces the surface area available to the enzymes.

In the work reported here, we had used wood chips from the stems of 6–7 yr-old *E. viminalis*. The stem wood of old trees is generally formed by a more heavily lignified tissue than that of young trees of the same species. Therefore, it seems probable that the fractionation of steam-treated substrates derived from hardwoods is influenced by the age of the tree. Earlier studies on the enzymatic hydrolysis of the steam-pretreated *E. regnans* derived from 40-yr-old stems indicated that alkali extraction resulted in a substantial decrease in the ease of hydrolysis of the water-insoluble substrate (*11*). We have previously shown that the sapwood region of the aspen wood stem is more permeable to steam than the heartwood region, probably because of the higher porosity of sapwood. Wood chips derived from young *E. viminalis* trees, which have a high proportion of sapwood present, are highly amenable to steam pretreatment and subsequent fractionation, resulting in a cellulosic residue which can be readily hydrolyzed to glucose.

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