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Organosolv-Pulping III

The Influence of Formic Acid Delignification on the Enzymatic Hydrolysis of *Pinus radiata* D. Don Sawdust

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ABSTRACT

Formic acid pretreatment on *Pinus radiata* D. Don was studied in order to improve the cellulose hydrolysis by commercial cellulase. The formic acid treatment effectively removed the lignin. A low substitution (formylation) and a crystallinity decrease of the cellulose in the pulp were observed. As consequence of these parameter changes, owing to the formic acid pretreatment on sawdust, a higher saccharification value was observed. The degree of saccharification increased when the degree of substitution (measured by titration) decreased and the portion of amorphous cellulose (measured via an X-ray technique) increased. *Trichoderma reesei* cellulase hydrolyzed the untreated and pretreated Pinus sawdust with formic acid in 25% and 56% of saccharification, respectively.

Index Entries: Organosolv pulping; enzymatic hydrolysis; formic acid delignification; *Pinus radiata.*

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INTRODUCTION

The world's chemical pulp is produced predominantly by the Kraft process and only a small part of its predecessor, the sulfite process. Recent improvement of the Kraft process (anthraquinone addition and extended cooking), although significant, do not solve any of the known basic problems related to the pollution problems (*1*). One possibility for overcoming existing problems is the use of an organic solvent instead of reacting inorganic chemical for the extraction of lignin from the lignocellulosic raw materials. The great conceptual advantage of using an organic solvent for lignin removal is that such a process offers the possibility for a more efficient utilization of the lignocellulosic feedstock (*2*).

Recently, we have developed a delignification procedure with formic acid (*3*) for use with *Pinus radiata* sawdust. This treatment was initially proposed by two German (*4,5*) and Canadian researchers (*6*), but for other types of wood. In 1983 the formic acid method was indicated as a convenient and economically feasible method for wood pulping, but no detailed technical description was published at that time (*7,8*). Now, we have established the conditions for delignification by formic acid treatment (*9*).

Very few studies on saccharification of *P. radiata* wood have been carried out. Among these are some pretreatments of the saccharification of *P. radiata* sawdust. Sodium hydroxide, milling and peracetic pretreatments gave a 16, 8, and 38% of total sugar, respectively (the untreated control with cellulase from *Aspergillus niger* (Sigma), 2.5 FPU/g sustrato was 6% in 30 h) (*10*). A value of 2.5% of saccharification after explosion pretreatment of *P. radiata* was obtained (in 48 h using Onosuka 3S cellulase) (*11*). In contrast, 33.6–52.6% of enzyme digestion of pulp was gained after explosion cooking with sulfur dioxide prior treatment of *P. radiata* woodchips (*11*). Optimization of this latter procedure yielded 82% of digestibility (*12*). However, in similar studies with Novozym 188, cellulase (*13*), and *Trichoderma reesei* C-30 cellulase (*14*), the saccharification values obtained in autohydrolysis-exploded *P. radiata* wood were lower.

Having in mind our previous results (*15–17*) in the delignification of pine sawdust and chips, in which the lignin was effectively removed after mild conditions (90°C, atmospheric pressure and low catalyst concentration) by treatment with concentrated formic acid in presence of HCl as catalyst, we have applied the formic acid pretreatment in the enzymatic hydrolysis of *P. radiata* sawdust by using cellulase complexes from *A. niger* and *T. reesei.*

MATERIALS AND METHODS

Pinus radiata D. Don sawdust was pretreated with formic acid as previously described (*3*). Commercial cellulases derived from *Trichoderma reesei* (Sigma Chem. Co. FPA=0.37 U/mg protein) and from *Aspergillus*

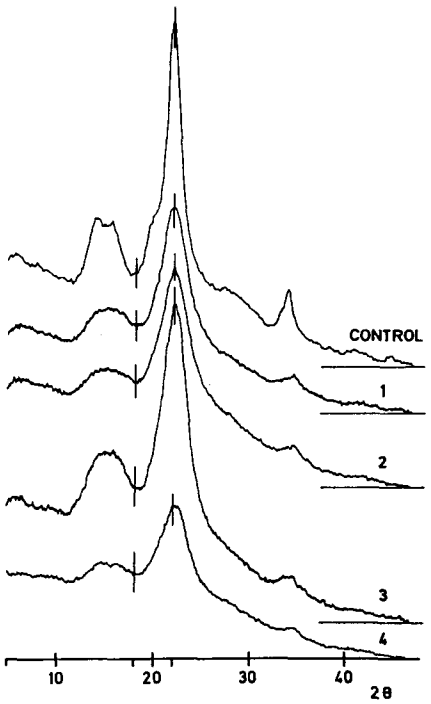

Fig. 1. X-ray difractogram. Control corresponds to crystalline cellulose, and the curves 1, 2, 3, 4 correspond to formic acid-pretreated sawdust using different catalyst concentrations: 0.5, 0.25, 0.125, and 0.0625 %, respectively.

niger (practical grade Type I, Sigma, FPA = 0.04 U/mg protein) and *T. reesei* Maxazyme CLH from Gist-Brochades, FPA = 0.32 U/mg protein). Degree of substitution (formylated) was determined according to the standard back-titration method (*18*).

The Debye-Scherrer diagrams were obtained on an X-ray Phillips Instruments PW 1140 Model (*19*). Crystalline index (CI) is defined as follows: $CI = \{(I_1 - I_2)/I_2\} \times 100$, where I_1 is intensity of difractogram at $2\Theta = 22.8°$ and I_2 is the intensity of difractogram at $2\Theta = 18°$ (Fig. 1). The proportion of amorphous substrate was determined as 100-CI (*20*). Cellulase activity was determined using the filter-paper reducing sugar test as described previously (*21,22*). Total reducing sugar were estimated using dinitrosalicyclic acid reagent (23). Cellulose content was measured by the method of Updegraff (*24*), and the Klason lignin by the standard method (*25*).

RESULTS AND DISCUSSION

One of the factors in the delignification process in formic acid solvation pulping considered was sawdust (g)/solvent (mL) ratio. The relative saccharification, the cellulose and Klason lignin contents, and the substi-

Table 1
Effect of Charge Ratio (g sawdust/mL Solvent)
on Pretreatment of Sawdust and its Saccharification[a]

Charge[b]	1/10	1/15	1/20	1/30
mg Sugar/mg substrate[c] $\times 10^2$	5.5	6.4	6.2	5.3
Relative Saccharification[c]	1.0	1.2	1.2	1.0
Cellulose Content (pulp) (%)	71	67	70	70
Klason Lignin (pulp) (%)	24	26	24	23
Hemicellulose (pulp) (%)[d]	5	7	6	7
Degree of Substitution	0.54	0.37	0.44	0.48

[a]Solvent: Formic acid-water-HCl(0.3%) (80:20:1, v/v) at 50°C, 2 h of extraction.
[b]Substrate concentration of 20 g/L.
[c]2.1 mg of *A. niger* cellulase at 50°C, pH 4.8, 0.05*M* citrate buffer at 2 h of incubation.
[d]by difference.

Table 2
Effect of Substrate Concentration on the Extent
of Conversion of Cellulose to Glucose at 1 H from *A. Niger* Cellulase[a]

Activity FPU/mg sawdust	Substrate Conc. g/L	mg Sugar/ mg substrate[b] $\times 10^2$	Saccharification[c,d] %
0.084	25	6.2	8.9
0.087	20	6.4	9.1
0.080	15	6.9	9.9

[a]Conditions at Table 1 at substrate/solvent charge of 1/15.
[b]70% of Cellulose.
[c][mg Sugar/mg substrate]/[mg of total carbohydrate/mg of substrate] $\times 100$.
[d]mg of Total carbohydrate/mg of substrate for the sample was 0.7.

tution degree related to the charge ratio, are shown in Table 1. No great difference on cellulose, Klason lignin, and hemicellulose content was observed in these pretreatments. The degree of substitution (formilation) changes slowly with the charge ratio, being 0.54 and 0.48 for the charges 1/10 and 1/30, respectively. Not great difference on the yields of the saccharification with the different substrates was observed, being a little more susceptible to saccharification the substrate obtained by using a ratio of 1/15, in which it is observed the lowest DS value. Presumably, the formylation value is an important fact in the saccharification of pretreated sawdust.

The effect of substrate concentration on saccharification was investigated with formic acid pretreatment of the *P. radiata* sawdust with *A. niger* cellulase at 0.08 FPU/mL. The results for 1-h digest are shown in Table 2. The extent of conversion of cellulose into glucose slightly increases as the substrate concentration decreases.

Table 3
Effect of Enzyme-Substrate Ratio on the Saccharification of Formic Acid Treatment on *Pinus Radiata* Sawdust[a]

FPU/mL	FPU/g Substrate	mg Sugar/mg Substrate $\times 10^2$	Saccharification[d] %
A. niger[b]			
0.04	3.0	4.1	5.8
0.09	6.0	4.1	5.8
0.14	9.0	5.2	7.5
T. reesei[c]			
0.36	24.0	6.5	9.3
0.80	53.0	10.1	14.4
1.40	93.0	14.2	20.3

[a]Conditions as Table 1, except that substrate concentration was 15 g/L.
[b]Inicial activity of *A niger* is 0.04 FPU/mg of solid.
[c]Inicial activity of *T. reesei* is 0.37 FPU/mg of solid, untreated control at 2 h, 30 h, and 60 h were 5.2, 9.2, and 25%, respectively.
[d]mg of Total carbohydrate/mg of substrate for the sample was 0.7.

The effect of cellulase (*T. reesei* and *A. niger*) concentration on the saccharification of sawdust from *P. radiata* at a substrate concentration of 15 g/L is shown in Table 3. Increasing cellulase concentration resulted in an increase in amount of total reducing sugar produced. An enzyme to substrate ratio of 9 FPU/g of sawdust gave 8% of saccharification after 60 h of hydrolysis for *A. niger*. However, increasing 2.6-fold the FPU/g substrate in *T. reesei*, only 9% of saccharification was reached. This is in agreement with the fact that cellulases that are synthesized by *T. reesei* are more active, comparatively with those characteristic of *A. niger*, and that the enzyme complex obtained from the latter contains a greater quantity of beta-glucosidase breaking down cellobiose and higher oligosaccharides to glucose (*26*).

The effect of temperature on the delignification process on saccharification is shown in Table 4. The substrate treated at 90°C with formic acid was more susceptible to enzymatic saccharification. Higher amounts of FPU of *T. reesei* gave higher saccharification values. Increasing the *A. niger* FPU units yielded markedly decreased saccharification. This is in agreement with the results obtained in Table 3.

At this stage, we have set up the best conditions of the enzymatic saccharification and temperature of the organosolv treatment (90°C), in which a high saccharification was afforded. Since a high Klason lignin (24%) in 80:20:1 solvent ratio of the sawdust treatment was detected in the residual solid materials, we decided to study different conditions of delignification in order to select a lignin pretreatment method to gain an optimal substrate for the enzymatic hydrolysis.

Table 4
Temperature Organosolv Cooking and Its Effect on Saccharification of *Pinus Radiata* D. Don Sawdust at Different *A. Niger/T. Reesei* Cellulase Ratio[a]

Temperature (°C) of Organosolv Procedure	mg Sugar/mg Substrate $\times 10^2$	Saccharification %
	A. niger/T. reesei (0.09)	
50	12.2	17.2
70	11.4	16.3
90	15.8	22.6
	A. niger/T. reesei (0.13)	
50	6.6	9.4
70	6.4	9.1
90	6.4	9.1

[a]At the same conditions on Table 1.

Table 5
Effect of Catalyst on the *Pinus Radiata* D. Don Sawdust After Formic Acid Treatment[a]

Conditions[b]	Residual Solid Yield, %	Klason Lignin, % (in the residual solid)	Cellulose, %
100:1	51	14	80
100:0.250	61	17	81
100:0.125	61	10	88
100:0.0625	65	8	91
90:10:1	67	21	73
80:20:1	75	24	75

[a]Original wood: 51.3% cellulose, 29.7% Klason lignin, 15.7% hemicellulose.
[b]Formic acid-H_2O-HCl (v/v), Sawdust/solvent charge 1/30 at 90°C for 10 min.

Results with different delignification conditions are showed in Table 5. Greater cellulose content and lower lignin content were obtained with 100:0.0625 charge ratio (formic acid:catalyst ratio), giving only a 8% of Klason lignin and 91% of cellulose on the residual solid material. Then, using pure formic acid instead of a formic acid-water mixture, better results were obtained in terms of recovery of the wood components.

Susceptibility to enzymatic degradation on the different source of cellulase was performed on the optimal substrate samples. Table 6 shows the effect of different amounts of cellulase of *A. niger* and *T. reesei*.

Again, *T. reesei* cellulase was more efficient in terms of reactivity and quality of the cellulase content than *A. niger*. With fourfold increase in the concentration with *A. niger* cellulase, only a small increase in the saccharification value was reached. However, in the case of *T. reesei*, the same increase of enzyme enhanced the saccharification by almost fourfold.

Table 6
Different Cellulase Sources on *Pinus Radiata*
D. Don Sawdust Previously Treated with Formic Acid[a,b]

Cellulase Source	FPU/mL	mg Sugar/ mg Substrate $\times 10^2$	Saccharification[c] %
A. niger	0.04	5.0	5.5
A. niger	0.16	5.6	5.9
T. reesei	0.40	8.0	9.0
T. reesei	1.50	28.0	31.0

[a]100:0.0625 Formic acid-catalyst ratio at 25 g/L of substrate and at 90°C for 1 h.
[b]As in Table 1.
[c]mg of Total carbohydrate/mg substrate was 0.91.

Thus, among all the treatments performed with different catalyst concentrations, 100:0.0625 was the most efficient delignification condition for the saccharification with *T. reesei* and *A. Niger*. We have tested a different cellulase, Maxazyme (FPU of 0.32 by mg protein) in a high FPU by milliliter of solution and compared with 100:0.250 conditions in which a different DS and an amorphous magnitude by X-rays were observed. Table 7 shows the effect of formic acid pretreatment of sawdust on structural features and saccharification value for 60 h.

In these conditions, a high value of saccharification in the 100:0.0625 formic acid catalyst ratio, as compared with 100:0.250 ratio, was obtained. This is in agreement with the fact that the residual solid material obtained had lower lignin content and high cellulose in the final residue. Also in this table, we can see that *T. reesei* cellulase is a better enzyme for pretreated sawdust for saccharification.

The relationship between saccharification rate and the set of structural features shows that a decrease in the crystallinity and lignin content enhances the saccharification value. Probably the high formylation value decreases the surface area of the substrate, thereby substantially decreasing the saccharification value. The specific surface area is the most influential structural feature, followed by the lignin content, which is in turn followed by the crystallinity of the substrate (*19,20*).

In conclusion, we find that the formic acid treatment used to remove the lignin and hemicellulose results in a lignocellulosic material accessible to enzymatic hydrolysis reactions. This pretreatment was more efficient than the milling and steam explosion methods (40%) previously applied to *P. radiata* wood (*13*). Only a very drastic pretreatment, such as explosion-cooking of sulfur dioxide-treated *P. radiata* wood (*12*), was more efficient than the formic acid treatment. However, such a procedure is more complicated and time consuming.

The carbohydrate fraction are now under study in order to obtain ethanol and single cell proteins from this pine sawdust.

Table 7
Effect of Formic Acid Pretreatment
of Sawdust in Structural Features and Saccharification[a,b]

	Residual yield, %	Klason lignin, %	Cellulose (residual solid) %	Hemicellulose (residual solid) %	Enzyme[c]	Saccharification %	DS[d]	Amorphous magnitude[e]
100:0.250	61	17	81	2	Maxazyme	31.0	0.66	46.9
100:0.0625	65	8	91	1	Maxazyme	34.0	0.49	54.8
100:0.0625	65	8	91	1	*T. reesei*	56.0	0.49	54.8
Untreated	-	29.7	51.3	15.7	*T. reesei*	25.0	-	-

[a] Substrate concentration: 15 g/l.
[b] Condition from Table 5.
[c] At 10 mg (3.2 FPU/mL) of Maxazyme and 12 mg (1.1 FPU/mL) of *T. reesei* incubated for 60 h following the method described in Table 1.
[d] Degree of substitution.
[e] 100-CI.

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