Role of surface charge and swelling on the action of xylanases on birch kraft pulp

Johanna Buchert, Maija Tenkanen, Maija Pitkänen, and Liisa Viikari

ABSTRACT: The swelling and the surface charge of birch kraft pulps affected the hydrolysis of pulp xylan with Trichoderma reesei *and* Bacillus circulans *xylanases. The more swollen the fiber, the less it was hydrolyzed. Furthermore, the greater the negative surface charge, the less the pulp was hydrolyzed. Both the swelling and the surface charge could be adjusted by suitable choice of counterions of the carboxyl groups in the pulp, indicating that the counterions in pulp have a profound effect on the action of xylanases in the pulp matrix.*

KEYWORDS: Betula, *bibliographies, carboxyl groups, kraft pulps, swelling, xylanases, zeta potential.*

The major polymeric constituents of wood—cellulose, hemicellulose, and lignin—contain various ionizable groups. These groups exhibit an acidic character, and a charge is generated if they can be ionized in the prevailing conditions *(1)*. Of the ionizable groups in pulp, only the carboxyl groups are ionized in neutral conditions *(1)*. The charges and the counterions of the carboxyl ions influence the polyelectrolytic behavior, the extent of swelling, and even the optical properties of the pulp, such as brightness stability *(2)*. The acidic-group content in wood is reported to be 60 mmol/kg or less *(3)*.

In kraft pulps, the majority of the carboxyl groups are methylglucuronic acid groups present in the xylan backbone *(1)*. In birch wood, the xylan is O-acetyl-4-O-methylglucuronoxylan, and on average, every tenth xylose unit is substituted by a methylglucuronic acid side group *(4)*. In pine wood, the xylan is mainly arabino-4-O-methylglucuronoxylan, and the amount of 4-O-methylglucuronic acid side groups is twice as high as in hardwood xylan. On average, two out of ten xylose units are substituted with uronic acid *(4)*.

During alkaline pulping, these acidic polysaccharides are partially dissolved because of the high pH and temperature *(5)*. As the cook proceeds, the alkali concentration decreases, allowing the degraded, short-chain xylan to precipitate in a more-or-less crystalline form on the surface of cellulose microfibrils *(6)*. In addition, the number of glucuronic acid side groups in hemicelluloses decreases because of alkaline hydrolysis of the glycosidic linkages of uronic acid units *(7, 8)*. New carboxyl groups are also generated during pulping because of the peeling reaction, which is halted by the formation of metasaccharinic acid or other alkali-stable carboxyl groups *(3)*. The first stages of chemical pulping also cause a rapid increase in the acidic group content through hydrolysis of carboxylic esters in wood *(9)*. Carboxyl groups are also introduced to the lignin fraction during kraft pulping, and the amount of carboxyl groups is about 20 carboxyl groups per 100 phenylpropane units *(1)*. After bleaching, the majority of the carboxyl groups in the bleached pulps are obviously 4-O-methylglucuronic acid groups *(10)*.

The acidic groups within the fiber wall are mainly responsible for the swelling properties of pulps in water. As a result of the dissociation of their counterions—the trace metals normally found in pulps—osmotic pressure is generated, causing water to penetrate into and swell the fibers. Swelling is more pronounced when the valency of the counterions is low, because osmotic pressure is dependent on the number of ions. Swelling can be

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decreased by high ionic strength *(9, 11)*. The number of dissociated counterions, which are kept in the vicinity of the carboxyl groups in the cell wall by electrostatic attraction, controls the osmotic pressure *(9)*.

Hemicellulases applied for a limited hydrolysis of hemicelluloses of kraft pulps have been found to enhance the extractability of lignins by conventional bleaching chemicals *(12–14)*. This effect is mainly due to the action of endo-ß-xylanases. Xylanases are believed to hydrolyze first the relocated, reprecipitated xylan on the surface of pulp fibers *(15)*.

Xylanases have been purified from various microorganisms, as reviewed by Viikari *et al. (16)*, and the enzymes differ from each other in their pI values, pH optima, and temperature stabilities. It has been suggested that the enzymatic hydrolysis of pulp xylan can be directed by chemical means, i.e., by adjustment of ionic strength and pH of the pulp solution *(17)*.

In this work, the effect of surface charge and swelling was studied with respect to enzymatic treatments. *Trichoderma reesei* and *Bacillus circulans* xylanases, with similar pI values but different pH optima, were chosen for this study.

Materials and methods

Kraft pulps

Birch kraft pulp (kappa no. 15.5) was obtained from a pulp mill. The carbohydrate composition of the pulp (expressed as percent of dry weight) was glucose 74.9%, xylose 24.2%, mannose 0.4%, arabinose 0, galactose 0.

Modification of the pulps

Pulps were modified using a slightly altered method of Scallan and Grignon *(18)*. Pulps were converted to acid form at 2% consistency in 0.1*M* HCl overnight at room temperature. The pulp was subsequently washed with distilled water and last with deionized (MilliQ) water until no chlorides were present in the washings. A drop of 50% $AgNO_3$ solution was added to a sample of the wash water, and the presence of chlorides was detected by formation of AgCl precipitate in the sample.

The acid pulp was converted to different metal forms in 0.1*M* metal chloride solutions (LiCl, KCl, $CaCl_2$, $BaCl_2$), and the pH of the suspensions was adjusted to 9.5 with the corresponding metal hydroxides. Aluminum pulp was produced by suspending the pulp in 0.25*M* $AlCl_3$ solution at 2% consistency without pH adjustment. Pulps were incubated at room temperature overnight with occasional shaking. Thereafter, the pulps were washed with distilled water and last with deionized (MilliQ) water until no chlorides were present in the washings. The pH values of the pulps were measured.

I. Metal content in modified pulps and calculated amount of metal-substituted carboxyl groups

Pulp counter-ion	Metal content, mg/kg								COOH,[a] mmol/kg
	Na	Ca	K	Mg	Mn	Al	Li	Ba	
Na	2200	20	200	10	<5	9	nd	nd	95.7
K	440	20	2900	30	<5	8	nd	nd	74.2
Li	100	10	70	10	<5	6	600	nd	86.5
Ca	<20	1900	<20	10	<5	<5	nd	nd	94.8
Ba	90	<10	30	10	<5	<5	nd	5800	84.5
Al	70	<10	30	<5	<5	1100	nd	nd	122.3
H	120	30	50	8	<5	<5	nd	nd	0
Ref.	2415	1084	294	227	87	14	nd	nd	113.0[b]

[a]COOH values express the calculated amount of metal-substituted carboxyl groups in the pulp.
[b]The carboxylic acid content of the reference pulp was measured by conductometric titration.

Modification of isolated birch xylan

Commercial glucuronoxylan from birch wood (Roth Art. 7500) was modified by an analogous procedure as used for the pulps. After conversion to different metal forms, the partly soluble xylans (only Al xylan was insoluble) were precipitated by adding two volumes of ethanol. Aqueous ethanol was also used for washing to remove the residual free ions. Finally, the modified xylans were lyophilized to dry powders.

Hydrolyses

A partly purified preparation of *B. circulans* xylanases (pI 8.8 and 8.4) *(19)*, obtained by ion-exchange chromatography in S-Sepharose FF, pH 5.2, and purified *T. reesei* xylanase (pI 9) *(20)*, were used in the enzymatic hydrolyses. The pulp treatments were carried out in deionized (MilliQ) water at 2% consistency. The enzyme dose was 500 nkat/g of pulp, and the hydrolyses were carried out for 1 h at 50°C. In the hydrolyses, the enzymes were dosed on the basis of equal activity units at each pH. Samples were centrifuged at 5000 rpm for 15 min and boiled for 2 min before analysis. With modified metal xylans, the substrate concentration was 1%, and the enzyme dose was 5000 nkat/g of xylan. The enzymes were also in this case dosed on the basis of equal activity units at each pH.

Inhibition of xylanases by metal chlorides

Inhibition tests were performed at 50°C in 50m*M* citrate buffer containing 100m*M* (NaCl, LiCl, KCl), 50m*M* ($BaCl_2$, $CaCl_2$) or 33m*M* ($AlCl_3$) of the metal chloride. The samples were incubated for 1 h, and the activities of the enzymes were measured using the XYL/DNS method *(21)*. The pH was 6.5 for *B. circulans* xylanase and 5.0 for *T. reesei* xylanase.

Analyses

Zeta potential was measured according to the microelectrophoresis principle using a Lazer-Zee-Meter (Model 400) *(22)*. Water-retention values (WRV) were measured according to the proposed SCAN method (SCAN-

II. Effect of counterions on swelling and zeta potential

Pulp counter-ion	WRV, %	Zeta potential, mV
Na	170	–47
K	169	–38
Li	172	–47
Ca	167	–26
Ba	168	–27
Al	164	–25
H	169	–29
Ref.	172	–33

III. Conditions in enzymatic hydrolysis

Pulp counter-ion	Enzyme	pH Initial	pH 1 hour
Na	*B. circulans*	8.0	7.1
K	*B. circulans*	7.2	7.1
Li	*B. circulans*	7.7	7.5
Ca	*B. circulans*	7.2	6.8
Ba	*B. circulans*	7.2	6.7
Al	*T. reesei* (pI 9)	5.1	4.4
H	*T. reesei* (pI 9)	4.2	4.3
Ref.	*T. reesei* (pI 9)	5.0	5.7
Ref.	*B. circulans*	6.5	7.0

M 102 X, proposal). Pulp composition was measured as described previously *(23)*. After the enzymatic treatment, the solubilized carbohydrates were analyzed as reducing sugars *(24)* or by HPLC (high-pressure liquid chromatography) after pretreatment with a mixture of xylanolytic enzymes *(25)*.

The metal concentrations of the pulps and isolated xylans were determined after dry ashing at 550°C in quartz crucibles. Lithium was determined by atomic emission spectrometry (PE 603). The other elements were determined by atomic absorption spectrometry using the graphite furnace technique (PE 5000/Z, HGA-400, AS-40) for aluminum, and the flame technique (PE 603) for sodium, potassium, calcium, magnesium, manganese, and barium. The carboxylic acids in the pulp were measured by conductometric titration *(26, 27)*.

Results

Modification of the pulps

The carboxylic acid content of the initial reference birch kraft pulp was 113 mmol/kg pulp, as seen in **Table I**. The carboxyl groups of pulps were first modified to metal-free acid form and thereafter converted to the various mono-, di-, and tri-valent counterion forms listed in Table I. By analyzing the concentration of the relevant cation in the pulp, the carboxylic acid content substituted with this cation could be calculated. The amounts of substituted carboxylic acids in the modified pulps were in the range of 74–122 mmol/kg pulp. This indicates that after modification of the pulp counterions, the carboxyl groups were rather completely substituted by the respective cations. Assuming that all the carboxyl groups originate from methylglucuronic acid groups in xylan, it can be calculated that the carboxylic acid content in xylan would be 300–500 mmol/kg xylan, corresponding to about 1:15 glucuronic acid group per xylose unit. An appreciable proportion of the uronic acid units present in the original hemicellulose are reported to survive kraft cooking and bleaching *(28)*.

The counterions of the carboxyl groups were found to have a pronounced role in the swelling and surface charge of the pulp, as seen in **Table II**. Although the differences in WRV were rather small, the values could be grouped according to the valency of the counterion of the carboxyl groups in different pulps. As expected, the surface charge (as measured by zeta potential) was most negative when the counterion was a monovalent metal ion. Similarly, these pulps had the highest swelling degree as measured by WRV. The WRV decreased in the modified pulps, and the zeta potential increased with increasing valency of the cations. These results are in accordance with results obtained by Scallan and Grignon *(18)*.

In the reference pulp, the major cations were sodium and calcium, their molar ratio being 3.9:1. The proportion of these cations in pulps is dependent on the process waters used in the mill *(29)*. The metal content of the reference pulp exceeded the carboxyl group content, indicating the presence of excess free metal cations in the pulp. The reference pulp had a WRV similar to that of the lithium pulp. The total metal content of the reference pulp was, however, much higher than that of the modified pulps, which may have affected the swelling. The zeta potential of the reference pulp was –33 mV and well between the values of sodium and calcium pulps.

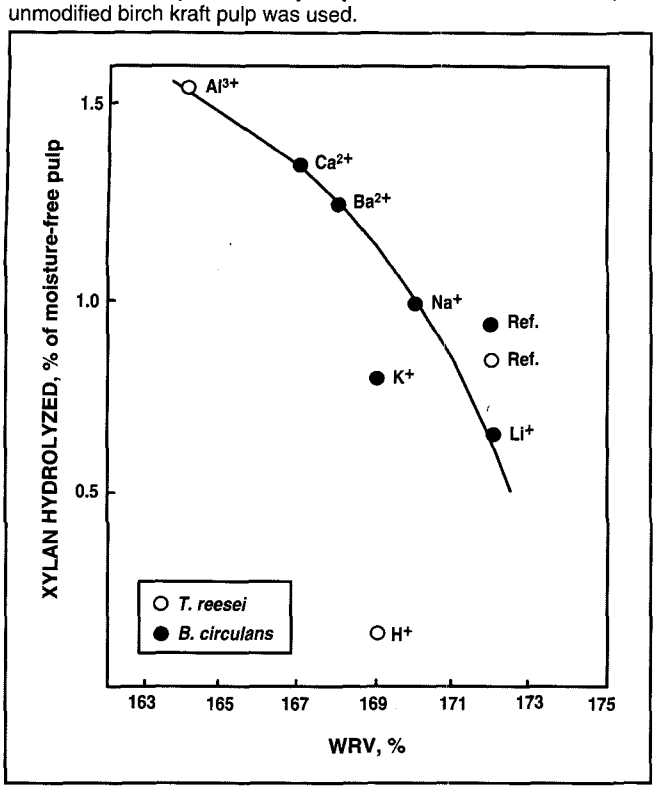
Effect of swelling and surface charge on xylanases

The modified pulps were used as substrate for the two xylanases isolated from *B. circulans* (pI 8.4 and 8.8) and the xylanase from *T. reesei* (pI 9). The *B. circulans* xylanases and the *T. reesei* xylanase had different pH optima *(19, 20)*. The pH values of the hydrolyses could not be adjusted because of the easy dissociation of the cations from the carboxyl groups. This is true especially for the monovalent metal cations below pH 7 *(30)*. However, the pH values were close to the pH optima of the enzymes, pH 7.2–8 for *B. circulans* and pH 4.2–5.1 for *T. reesei* xylanases, as seen in **Table III**.

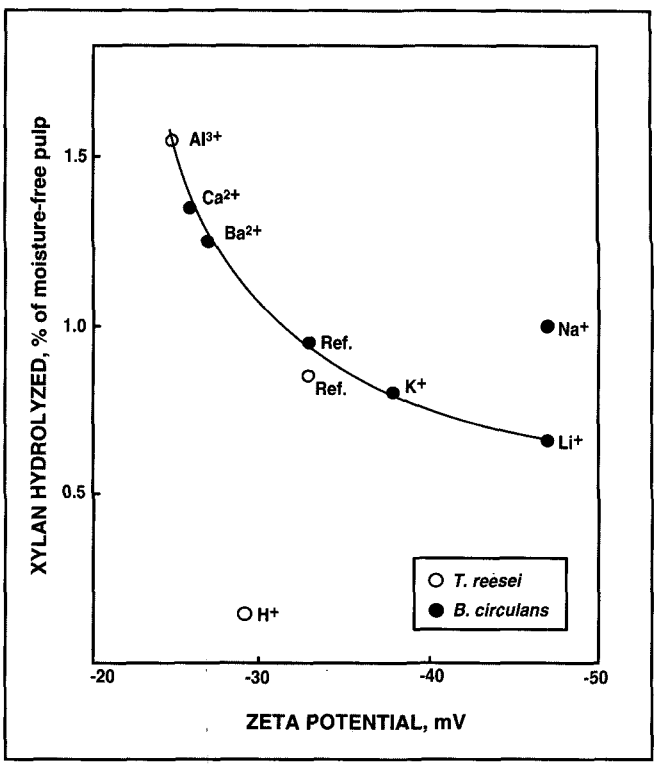
Although the differences in the WRV values of the pulps were relatively small, it appeared that the more swollen the fiber was, the less it was hydrolyzed, as illustrated in **Fig. 1**. Surprisingly, the metal-free pulp was poorly hydrolyzed by the *T. reesei* xylanase (Fig. 1), although the pH of the hydrolysis was in the optimal range of this enzyme *(20)*. Similar results have been observed with other xylanases (results not shown).

Interestingly, it was also observed that the surface charge (as measured by zeta potential) had a profound effect on the hydrolysis of xylan from the fiber, as seen in **Fig. 2**. The less negative the charge was, the higher was the degree of hy-

1. Effect of swelling on the hydrolysis of birch kraft pulp with *T. reesei* and *B. circulans* xylanases. Hydrolysis time 1 h. As a reference, an unmodified birch kraft pulp was used.



2. Effect of surface charge on the hydrolsysis of birch kraft pulp with *T. reesei* and *B. circulans* xylanases



IV. Effect of metal chlorides on the activity of xylanases

Metal chloride	Concentration, mM	Activity, % T. reesei	Activity, % B.circulans
LiCl	100	133	100
NaCl	100	96	96
KCl	100	95	94
$CaCl_2$	50	65	109
$BaCl_2$	50	84	111
$AlCl_3$	33	1	4
Ref.*	...	100	100

*Citrate buffer, 50m*M*

V. Metal content in substituted xylans and calculated amount of metal-substituted carboxyl groups

Metal xylan	Metal content, mg/kg Na	Ca	K	Al	Li	Ba	COOH,* mmol/kg
Na-xylan	12,000	330	300	490	nd	nd	520
K-xylan	80	140	21,000	14	nd	nd	540
Li-xylan	200	210	140	21	4,500	nd	650
Ca-xylan	<20	11,000	<20	60	nd	nd	550
Ba-xylan	<100	<100	<100	19	nd	48,000	700
Al-xylan	<20	30	<20	4,400	nd	nd	490
H-xylan	150	<20	190	10	nd	nd	(14)
Ref. (Roth)	39,000	2,500	200	14	nd	nd	nd

*COOH values express the calculated amount of metal-substituted carboxyl groups in xylan.

drolysis. The hydrolysis yield increased twofold when zeta potential increased from -47 mV to -25 mV.

The role of the metal chlorides on the activity of the xylanases was also tested, because the metal ions, as such, may also cause inhibition of the enzymes. Both xylanases were strongly inhibited by aluminum chloride, as seen in **Table IV**. This was surprising, since aluminum pulp was most readily hydrolyzed by the *T. reesei* xylanase. The activity of *T. reesei* xylanase (pI 9) was also found to be slightly inhibited by $CaCl_2$. *B. circulans* xylanase was inhibited only by aluminum. Other cations did not affect its activity, although monovalent pulps were less easily hydrolyzed than the divalent pulps.

The hydrolysis of isolated xylans converted to different metal forms was also studied in order to clarify further the effect of the metal-substituted substrate on the action of the enzymes.

VI. Hydrolysis of the metal-substituted hardwood xylans with *B. circulans* and *T. reesei* xylanases

Metal xylan	pH	Hydrolyzed portion, % T. reesei	Hydrolyzed portion, % B. circulans
Na-xylan	7.5	22.1	20.2
K-xylan	7.1	0.5	10.6
Li-xylan	6.9	17.8	16.4
Ca-xylan	6.7	22.8	21.7
Ba-xylan	7.4	21.6	20.6
Al-xylan	3.9	19.7	12.2
H-xylan	3.4	1.8	6.5
Ref.	5.4	22.4	22.0

The total metal-substituted carboxylic acid content of the modified xylans was in the range of 500–700 mmol/kg xylan, corresponding well to the values obtained in pulp, as seen in **Table V.** Thus, this glucuronoxylan apparently has carboxylic acid groups in an amount similar to that of the xylan in birch pulp.

No great differences were observed in the hydrolyses, except that only potassium xylan was poorly hydrolyzed by both *T. reesei* and *B. circulans* xylanases, as seen in **Table VI.** As such, the potassium ion did not affect the activity of the enzymes, but when the potassium was bound to the xylan substrate, the enzymatic reaction was restricted. The other cation-substituted xylans were readily hydrolyzed by both of the enzymes, indicating that the poor hydrolyzability of monovalent-cation pulps (lithium or sodium) is not caused by the metal cation. The differences in behavior are merely caused by physical parameters, such as swelling or surface charge. The metal-free xylan was also poorly hydrolyzed (Table VI), further confirming the important role of metal counterions of carboxyl groups in the action of xylanases.

Discussion

The swelling and the surface charge of birch kraft pulps were found to affect the action of xylanases on pulps. The more swollen the fiber was, the less it was hydrolyzed. Furthermore, the more negative the surface charge was, the less the pulp was hydrolyzed. Both the swelling and the surface charge could be adjusted by suitable choice of the metal counterions of the carboxyl groups in pulp, indicating that these counterions in pulp have a profound effect on the action of xylanases in the pulp matrix. The observed differences in the hydrolysis of pulp xylan may also be caused by the intrinsic pH of the fibers, which may be affected by the degree of dissociation of the counterions.

According to the proposed mechanism of enzyme-aided bleaching, xylanases are believed to act mainly on the reprecipitated xylan located on the surface on the fibers *(15)*. The actual amount of reprecipitated xylan in kraft pulps is not known, but it has been suggested that half of the xylan present in pine kraft pulp is relocated xylan *(6, 31)*. In birch kraft pulp, the reprecipitated xylan has been estimated to be about 5–10% of the total xylan in the pulp *(32)*. The carboxylic acid content of this reprecipitated xylan is also unknown. The location of the carboxylic acid within the fibers is interesting. Either they are universally distributed around the fibers, or the reprecipitated xylan is less acidic. The distribution of acidic groups within the fibers can be expected to have a pronounced effect on the action of enzymes.

This important effect of surface charge and swelling has not previously been studied with respect to the action of enzymes on the fibers. The role of these interactions is under further investigation. These parameters are expected to have a pronounced significance for applications such as enzyme-aided bleaching. The chemical environment (pH and various metal ions) during beating and sheet forming is already known to influence the final paper properties *(18, 33)*. Therefore, modification and optimization of these parameters may also expedite the action of enzymes in enzyme-aided bleaching or other enzymatic applications in the pulp and paper industry.

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