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Stability of xylanases in the presence of methanol and its evaluation on the bleaching capacity

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Abstract

A commercial (Cartazyme) and non-commercial (Asperzyme) xylanases were studied. Cartazyme stability in a 0–70% (v/v) methanol at 50 °C and 65 °C was carried out. No deactivation was found for Cartazyme in the presence of 15% methanol at 50 °C. Half-life activity decay ($t_{1/2}$) of Cartazyme at 50 °C in 30%, 50% and 70% methanol solutions were 4.0 h, 2.3 h and 1.2 h, respectively. At 65 °C, which is the ozone-alkali-peroxide (ZEP) bleaching temperature, only significant results on Kappa number reduction and selectivity were only observed in 15% methanol ($t_{1/2}$ 30 min) at the Z stage. For the Asperzyme, a $t_{1/2}$ of 36.5 min at 50 °C was found. In the Z stage with Asperzyme in the presence of 25% of methanol, a 20% Kappa number reduction and an improvement of the ZEP sequence of the brightness of 3.1 points were obtained. These results were correlated with the xylanase stability.

Introduction

In recent years xylanases have received much attention due to their possible application in the pulp and paper industry such as prebleaching of kraft pulp, debarking, refining pulp fibers and preparing dissolving pulps (Eriksson 1997, Srebotnik & Messner 1996, Prade 1996). The applications of xylanases in the bleaching of *Pinus radiata* D Don kraft pulp have been described (Aguirre *et al.* 1995, Allison *et al.* 1995, Curotto *et al.* 1993, 1998, Durán *et al.* 1994, Kibblewhite & Clark 1996, Ruiz *et al.* 1993, Vicuña *et al.* 1995). Some experiments on *Pinus radiata* D Don totally chlorine free (TCF) pulps with xylanases were carried out (Ruiz *et al.* 1993, Durán *et al.* 1994, Aguirre *et al.* 1995, Vicuña *et al.* 1995, Angelo & Durán 1998a,b), but very little is known about the direct use of xylanase in organic solvent and subsequent TCF bleaching. Enzyme catalysis in aqueous-organic co-solvent mixtures has wide applications. However, not enough attention has been paid to the issue of stability of enzymes in such media (Gupta *et al.* 1997). Xylanases are quite stable after precipitation with ethanol (Bakalova *et al.* 1995, Tan *et al.* 1993, Ganga *et al.* 1997). In the presence of ethanol, an increase of the activity at 5% ethanol was found and a significant decrease at a high concentration was observed (Herrmann & Kubicek 1996). The activity was decreased by addition of methanol and ethanol at the concentration of 10% on xylanase from *Aspergillus niger* (Sung *et al.* 1996).

The aim of this research was to understand better the stability of xylanase in the presence of methanol and the evaluation of its potential use in pre-bleaching using the same solvent on a direct TCF bleaching in a ozone-alkaline extraction-peroxide (ZEP) sequence.

Table 1. Decay constants (kd) and half-lives ($t_{D0.5}$ of xylanase (Cartazyme) at different methanol concentrations[a].

Temperature		Methanol (% v/v)				
		0	15	30	50	70
50 °C	kd ($\times 10^{-3}$ min^{-1})	4.5[b]	ND	2.9	5.0	9.7
	$t_{1/2}$ (min)	150.0	ND	240.0	138.0	71.0
65 °C	kd ($\times 10^{-3}$ min^{-1})	11.3	23.1	46.0	120.0	242.0
	$t_{1/2}$ (min)	61.0	30.0	15.0	6.0	3.0

[a] Average deviation in triplicated was less than 5%.
[b] ND: no heat denaturation for 1 h.

Table 2. Effect of methanol in the ozone stage (Z stage) (0.6%/b.d.pulp, pH 2.5) after Xylanase treatment (Cartazyme) (1 h, 65 °C).

Treatment (65 °C)	Kappa number	Viscosity (cps)	Selectivity[a]
Control	8.9	15.2	0.9
Xylanase(X)[c]	8.4 (6%)[b]	16.0 (5%)	1.0
X/15% methanol (XM15)	8.0 (16%)	19.2 (21%)	1.5
X/30% methanol (XM30)	8.0 (7%)	17.2 (13%)	1.3
X/50% methanol (XM50)	8.1 (0%)	20.5 (26%)	1.8
X/70% methanol (XM70)	8.6 (0%)	18.3 (17%)	1.3
15% methanol (M15)	9.15(−6%)	20.4 (26%)	1.4
30% methanol (M30)	8.6 (3%)	19.6 (23%)	1.5
50% methanol (M50)	8.1 (9%)	19.5 (17%)	1.6
70% methanol (M70)	8.3 (7%)	18.0 (16%)	1.3

[a] Selectivity is the degree of carbohydrate degradation and is usually known as the reduction of pulp viscosity when pulp is bleached to a certain kappa number level. Selectivity = Kappa number reduction (%)/viscosity reduction (%). Original pulp characteristics: Kappa number: 15; viscosity: 27.7, brightness: 40.5%.
[b] In parenthesis % reduction (Kappa) or% enhancement (viscosity) related to the control experiment.
[c] X= xylanase in aqueous solution; X/15% methanol (XM15) = xylanase in 15% methanol-aqueous solution; the rest of symbols correspond to different methanol-aqueous solutions.

Table 3. Effect of methanol in the ozone-basic extraction-peroxide (ZEP sequence) (Cartazyme).

Treatment (65 °C)	Kappa number	Viscosity (cps)	Selectivity	Brighness (%)
Control	4.2	14.0	1.5	73
X	3.8	14.3	1.5	74
XM15	4.3 (0%)	16.0 (13%)	1.7	74
XM30	4.1 (0%)	14.3 (0%)	1.5	75
XM50	3.5 (17%)	17.5 (20%)	2.1	75
XM70	3.3 (21%)	15.6 (10%)	1.8	76
M15	4.3 (0%)	15.4 (9%)	1.6	74
M30	4.1 (0%)	16.3 (14%)	1.8	71
M50	3.3 (21%)	17.6 (20%)	2.1	75
M70	3.4 (19%)	15.6 (10%)	1.8	75

[a] Conditions as in Table 2.

Table 4. Decay constants (kd) and half-lives ($t_{D0.5}$) of xylanase (Asperzyme) at different methanol concentrations[a].

Temperature		Methanol (% v/v)						
	0	10	20	30	40	50	60	
40 °C	kd ($\times 10^{-3}$ min^{-1})	1.6	1.4	2.5	3.6	7.1	17.0	48.0
	$t_{1/2}$ (min)	433.0	495.0	277.0	192.0	97.6	40.1	14.4
50 °C	kd ($\times 10^{-3}$ min^{-1})	19.0	31.0	47.0	64.0	98.0	120.0	340.0
	$t_{1/2}$ (min)	36.5	22.3	14.7	10.8	7.1	5.8	2.0

[a]Average deviation in triplicated was less than 5%.

Table 5. Effect of methanol in the Z and ZEP sequences (Asperzyme).

Stage	Procedure	Kappa number	Viscosity (cps)	Selectivity	Brighness (%)
Z	Control	5.0	11.5	1.14	–
Z	X	4.4 (12%)	11.2 (0%)	1.18	–
Z	XM 25	4.0 (20%)	13.7 (16%)	1.45	–
Z	XM50	5.0 (0%)	11.9 (9%)	1.17	–
Z	M25	5.0 (0%)	–	–	–
Z	M50	4.6 (9%)	–	–	–
ZEP	Control	1.1	11.4	1.58	83.7
ZEP	X	1.0 (9%)	10.0 (−13%)	1.46	82.5
ZEP	XM25	1.1 (0%)	11.8 (3%	1.62	86.8
ZEP	XM50	1.0 (0%)	11.3 (0%)	1.60	86.2
ZEP	M25	1.1 (0%)	–	–	–
ZEP	M50	0.9 (2%)	–	–	–

[a]Initial Kappa number: 15.0; Viscosity: 27.7 cps; Brightness: 40.5. Enzyme treatment was carried out at 50 °C, pH 5.5, charge 8 IU g^{-1}, 1 h reaction time, consistence 8%.

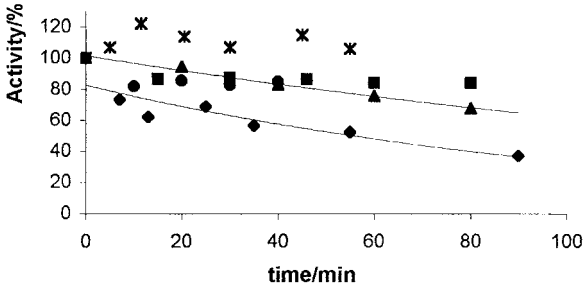

Fig. 1. Heat denaturation of xylanase at 50 °C at initial activity of 4 IU ml^{-1} at pH 6.1 at different methanol concentrations: H2O (●), M15 (15%) (⋆) , M30 (30%) (■), M50 (50%) (▲) and M70 (70%) (♦).

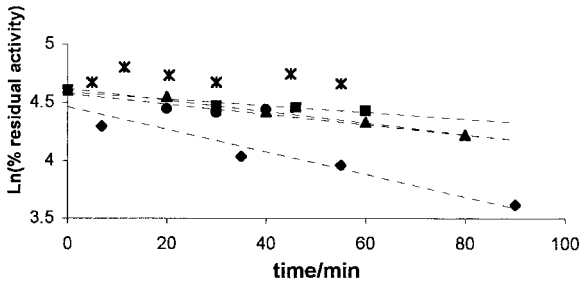

Fig. 2. Heat denaturation of Cartazyme at 50 °C at different methanol concentrations: H2O (●), M15 (15%) (⋆), M30 (30%) (■), M50 (50%) (▲) and M70 (70%) (♦).

Material and methods

Pulp. Extended cooked oxygen-delignified softwood kraft pulp of *Pinus radiata* with Kappa number of 15.0, viscosity of 27.7 Cps and brightness of 40.5% was employed.

Enzymatic treatment. Pulps were incubated with a commercial xylanase (Cartazyme 9704E) under the following conditions: 3 IU g^{-1}, 65 °C, pH 6.0, 3 h at different concentrations of methanol (0–70% vv^{-1}). The pH of the mixture was adjusted by adding 2 mol l^{-1} sulfuric acid. Incubation was conducted for 3 h in a rotavapor containing 150 g of pulp at 10% consistency. After the incubation step, pulps were im-

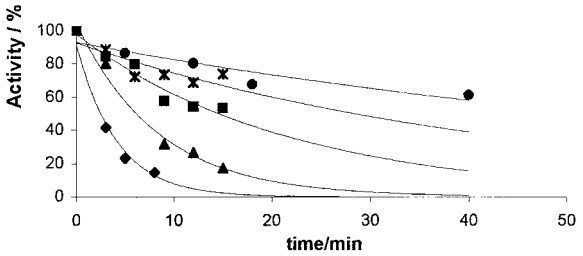

Fig. 3. Heat denaturation of xylanase at 65 °C at initial activity of 4 IU ml^{-1} at pH 6.1 at different methanol concentrations: H2O (●), M15 (15%) (⋆), M30 (30%) (■), M50 (50%) (▲) and M70 (70%) (♦).

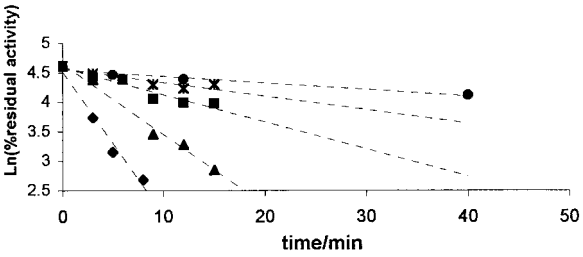

Fig. 4. Heat denaturation of Cartazyme at 65 °C at different methanol concentrations: H2O (●), M15 (15%) (⋆) , M30 (30%) (■), M50 (50%) (▲) and M70 (70%) (♦).

mediately treated with a chemical bleaching sequence in an ozone reactor. Pulps were also treated with Asperzyme 2M1 (Angelo *et al.* 1997, Curotto *et al.* 1998) under the following conditions: 8 IU g^{-1}, 50 °C, pH 5.5, 1 h at 25% methanol.

Chemical bleaching. Ozone treatments at high pulp consistency (40% w/v) were according to laboratory procedures employed previously (Ruiz *et al.* 1997). Prior to ozonization, pulps were soaked at pH 2.5 (in the same solvent-mixture used for the enzymatic treatment) for 1 h, excess solvent was then pressed out and the mixture centrifuged to obtain the desired pulp consistency. These impregnated pulps were fluffed and ozonized in a rotating round-bottomed flask equipped with a gas inlet system; the ozone charge applied was of 0.6%/b.d. pulp.

The alkaline extraction stage was performed at 10% consistency in a polyethylene bag, at 70 °C, during 60 min, with a NaOH charge of 2%. The hydrogen peroxide stage was performed at 10% consistency in a polyethylene bag, at 70 °C during 180 min. The necessary amount of 0.1 M NaOH was added to reach pH 11.5. Hydrogen peroxide, magnesium sulfate an silicate concentrations were 1%, 0.5% and 1%, respectively.

Pulp analysis. Measurements of Kappa number, viscosity and brightness were performed according to TAPPI methods.

Xylanase stability. Five milliliters aliquot of the enzyme solution were placed in a test tubes in a water bath at the desired temperature (± 0.5 °C) and periodically shaken at the pH 6.1 after 0–70% (v/v) methanol addition. The reported times for the denaturation studies correspond to the times at which the enzyme samples were removed from the test tubes in the water bath. Then the enzyme mixture was allowed to equilibrate to the adequate temperature for measurement the xylanase activity following the standard Bailey's method (Bailey *et al.* 1992).

Results and discussion

Denaturation profiles for xylanase in the presence of methanol in different concentrations are shown in Figures 1 and 2 (50 °C), and in Figures 3 and 4 (65 °C). At all temperatures, the heat-denaturation kinetics can be described as first-order. Half-lives in the presence of 15% methanol, ranging from no-deactivation at 50 °C to 30 min at 65 °C (Table 1), at pH 6.1 were observed. In the presence of 30% methanol, the half-lives ranged from 4 h at 50 °C to 15 min at 65 °C. A low stability in 50% methanol at 50° and 65 °C with half-lives of 2.5 h and 6 min, respectively, were found. The Cartazyme appeared with a excellent heat-denature stability in the presence of methanol, since the maximum time of treatment is 1.0 to 1.5 h between 50–65 °C (Figures 2 and 4).

In 15% methanol and at 65 °C, a $t_{1/2}$ a 0.5 h was attained, however, with 30 to 70% methanol a very rapid denaturation of 15–2.9 min was observed. This indicates that, for instant, in 30% of methanol 4 IU ml^{-1} in 15 min decreased to 2.8 IU ml^{-1} and after 3 h treatment only 0.001 IU ml^{-1} will be present in the pre-treatment stage. This enzyme denaturation is clearly observed in Table 2, where the pulp was analyzed after ozone bleaching.

In terms of Kappa number in the ozone stage, a 6% of kappa reduction in the presence of xylanase and absence of methanol was found. In the presence of 15% methanol (M15), an inhibition of the delignification with ozone was observed. However, in the presence of xylanase (XM15), a 16% of Kappa reduction together with a 21% of viscosity protection were observed. These results indicate that the xylanase and

methanol exert a positive effect on the pulp quality. This is in agreement with the xylanase stability at this temperature. On increasing the methanol concentration, a rapid denaturation at 65 °C was observed and only the methanol effect on the ozonization stage was observed. Also, the selectivity at XM15 was higher than that of methanol alone.

At the final stage of TCF bleaching (ZEP), Table 3 shows a 10% of Kappa number reduction in the pre-treatment with xylanase. However, no change in the presence of 15% of methanol was found. However, an enhancement in the viscosity protection of 13% of XM15 over only 9% in M15 were observed. Selectivity in the XM15 slightly enhanced over M15, keeping the same brightness values. The rest of the experiments show the methanol effect on the bleaching process.

Then in the case of Asperzyme, the apparent thermal stability constant in the presence of methanol (0–60%) at 40 °C and 50 °C were measured. The $t_{1/2}$ was found to vary between 14.4 and 433.0 min at 40 °C and between 2.0 and 36.5 min for the assays at 50 °C. The residual activities were measured using birch xylan as substrate. These stabilities were lower than those in the Cartazyme experiments since in the latter, the presence of a stabilizer lead to an increase of $t_{1/2}$ for this enzyme. In the experiments with Asperzyme, no stabilizer was added in order to only study the enzymatic effect on the pulp. Previously, it was shown that 50% glycerol increases the thermal stability from a half-life of 7.2 min to 2.6 h at 55 °C (Angelo *et al.* 1998).

In order to obtain the best results on the methanol and temperature effect (50 °C), in a reasonable period of time (e.g., 1 h), an intermediate methanol concentration (25% v/v) and 50% methanol were selected for the pulp treatment. In these conditions, $t_{1/2}$ was approximately 12 min (25% methanol) and 6 min (50% methanol). Although, the relative low $t_{1/2}$ at 50 °C, these lifetimes should be enough to improve the final pulp quality as it was demonstrated in Table 5, specially in the Z stage with XM25.

In the experiment with Asperzyme 2M1 at the Z stage in the absence of methanol (Table 5), a 12% of Kappa number reduction was observed. However, in the presence of 25% of methanol, a 20% of Kappa number reduction and an improvement of viscosity of 2.2 points at the Z stage were observed. At the ZEP stage, the xylanase treatment in the presence and absence of methanol did not change the Kappa number and the viscosity values. However, the brightness significantly was improved at the X-stage (3.1 points).

Conclusions

In view of these results and the xylanase stability, 65 °C for the Cartazyme 9704E applied to a ZEP sequence is not adequate. The indication from these results is to decrease the temperature of the xylanase pre-treatment between 50–55 °C in order to increase the half-live of the enzymatic stability and improve the results. In the ozone stage, a 15% methanol-aqueous mixture was the most efficient. A good result, in spite of the low $t_{1/2}$ of enzyme at 50 °C, of brightness and selectivity values with the Asperzyme was obtained. In this case, an addition of stabilizer is indicated in order to obtain an optimal stability of the enzyme for the pulp improvement (Angelo *et al.* 1997). The approximate price of xylanase in 1996 was US $ 2 per ton and a calculation of relative economic benefits in an elemental chlorine free process (ECF) sequence reveals that the reduction of 5 kg ClO_2/ton of pulp leads to saving of about US$ 2 per ton of pulp in chlorine dioxide cost alone. In a TCF process the cost of oxygen based chemicals (ozone, peroxide) are even higher and the respective saving even more pronounced (Viikari *et al.* 1997). Studies on the fibers qualities (Durán & Angelo 1998) and an optimization of the process is actually in progress (Baeza *et al.* 1999).

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