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Effect of pH on production of xylanase by *Trichoderma reesei* on xylan- and cellulose-based media

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Abstract. *Trichoderma reesei* VTT-D-86271 (Rut C-30) was cultivated on media based on cellulose and xylan as the main carbon source in fermentors with different pH minimum controls. Production of xylanase was favoured by a rather high pH minimum control between 6.0 and 7.0 on both cellulose- and xylan-based media. Although xylanase was produced efficiently on cellulose as well as on xylan as the carbon source, significant production of cellulase was observed only on the cellulose-based medium and best production was at lower pH (4.0 minimum). Production of xylanase at pH 7.0 was shown to be dependent on the nature of the xylan in the cultivation medium but was independent of other organic components. Best production of xylanase was observed on insoluble, unsubstituted beech xylan at pH 7.0. Similar results were obtained in laboratory and pilot (200-l) fermentors. Downstream processing of the xylanase-rich, low-cellulase culture filtrate presented no technical problems despite apparent autolysis of the fungus at the high pH. Enzyme produced in the 200-l pilot fermentor was shown to be suitable for use in enzyme-aided bleaching of kraft pulp. Due to the high xylanase/cellulase ratio of enzyme activities in the culture filtrate, pretreatment for removal of cellulase activity prior to pulp bleaching was unnecessary.

Introduction

Trichoderma spp., particularly *T. reesei*, are well known as producers of both cellulolytic (Ryu and Mandels 1980; Nevalainen et al. 1990) and xylanolytic enzymes (Wong and Saddler 1992). Although the results of some early studies on enzyme production by mutant strains of *T. reesei* were interpreted as indicating a common regulatory mechanism for production of cellulases and xylanases (Nevalainen and Palva 1978), later work has shown that separate induction mechanisms are also involved both in *T. reesei* (Hrmová et al. 1986) and in *T. longibrachiatum* (Royer and Nakas 1990). Confusion may be caused by the fact that some enzymes, e.g. endoglucanase I of *T. reesei*, may harbour both xylanolytic and cellulolytic activities (Bailey et al. 1993).

As a result of recent successes in the application of xylanases in enzyme-aided bleaching of kraft pulps (Viikari et al. 1986, 1991; Koponen 1991), xylanases have acquired major potential as bulk enzymes for large-scale industrial applications. An important quality criterion for xylanase preparations for use in the pulp industry is very low cellulase activity. Some results have been published concerning removal of cellulase from mixed-activity culture filtrates of *T. harzianum* during downstream processing (Tan et al. 1987, 1988). However, a more realistic approach would probably be to use microorganisms and processes producing high levels of xylanase with only low cellulase activity or by genetically modifying strains to overproduce xylanase without production of cellulase activity (Suominen et al. 1992). Examples of xylanase-producing organisms with low cellulase activity include some species of *Aspergillus* (Biely 1985; Bailey and Poutanen 1989; Bailey and Viikari 1993) and hyperproducing mutants of the yeast-like fungus *Aureobasidium pullulans* (Leathers et al. 1984, Leathers 1989).

Another alternative for the production of cellulase-free xylanase is provided by the knowledge that production of xylanases and cellulases by *Trichoderma* is under separate regulatory control. Dekker (1983) first reported that xylanase production by *T. reesei* was dependent on the pH of the culture, although the activity levels obtained in this work were all very low. Royer and Nakas (1989) confirmed that high pH (>6.0) was an important factor for xylanase production by *T. longibrachiatum*, although cellulase production, e.g. by *T. reesei*, is usually carried out at lower pH (e.g. Montenecourt and Eveleigh 1979; Bailey and Nevalainen 1981). Royer and Nakas (1989) obtained best xylanase production on media containing cellulose as the carbon

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source, with considerably poorer production on xylan. Very recently Gamerith et al. (1992) obtained rather high xylanase production by *T. reesei* on xylan-based substrates, but these authors did not investigate the effect of pH on enzyme production.

The aim of the present work was to investigate the effects of pH and medium composition on the production of xylanase and cellulase by *T. reesei* VTT-D-86271 (Rut C-30).

Materials and methods

Microbial strain. T. reesei VTT-D-86271 (Rut C 30) was maintained during the investigation on potato dextrose agar slants under a daylight bulb at room temperature, with subculturing at intervals of 2–4 weeks.

Shake-flask cultivations. These were carried out using 50 ml medium in 250-ml flasks on an elliptical-orbit shaker at 200 rpm and 29° C. The carbon sources tested were Solka floc cellulose (James River Corporation, N. H., USA), insoluble, essentially unsubstituted beech xylan (Lenz and Schurz 1986, obtained from Lenzing, Austria), soluble deacetylated birch 4-*O*-Me-glucuronoxylan (Roth 7500), wheat bran and acetylated xylo-oligosaccharides from steaming of birch wood by the Stake process (obtained from Cultor, Finland). All carbon sources were tested at a concentration of 20 g l^{-1} with 10 g l^{-1} of either distiller's spent (wheat) grain (SG) or spray-dried corn steep solids (CSS) as a source of organic nitrogen and trace nutrients. The media contained KH_2PO_4 (15 g l^{-1}) and $(NH_4)_2SO_4$ (5 g l^{-1}), providing some buffering power and an initial pH after sterilization of 4.4±0.2 without adjustment.

Fermenter cultivations. Most of the fermenter cultivations were carried out in a Chemap CF 3000 fermentor, total working volume 10 l including the 1-l inoculum grown on the appropriate carbon source at 30 g l^{-1} in two stages (1×200 ml for 2 days and 5×200 ml for 1 day). Some cultivations were also carried out in minifermenters, working volume 1300 ml including a 100 ml inoculum grown for 2 days. The fermentation conditions were: temperature, 29° C; pH controlled by automatic addition of NH_4OH or H_3PO_4; $pO_2 \geq 20\%$, controlled by agitation; aeration, 0.5 l l^{-1} min^{-1}. Media used in the fermenter cultivations were 60 g l^{-1} Solka floc cellulose with 30 g l^{-1} of SG, or 30 g l^{-1} of beech xylan (Lenz and Schurz 1986) with 15 g l^{-1} of CSS. Higher concentrations of the beech xylan were not practical because of excessive broth viscosity. Other medium components were KH_2PO_4 and $(NH_4)_2SO_4$, both at 5 g l^{-1}. Foaming was controlled by addition of Glanopon (Schill and Scheilacher, Hamburg, Germany). In-place sterilization was for 15 min at 121° C.

A pilot cultivation was carried out in the 200-l pilot fermenter at this laboratory. The medium contained 30 g l^{-1} of beech xylan and 15 g l^{-1} of CSS as described above, and the cultivation conditions were: temperature, 29° C; pH > 7.0; agitation, 200 rpm. After the cultivation, the broth was separated on a vacuum drum filter (Larox, Finland, model VF 8, surface area 1.0 m^2) and concentrated by ultrafiltration using PCI ES 625 membranes (nominal cut-off 25 kDa, $A = 2.4$ m^2, pumping pressure 7.5 bar/20 l min^{-1}, $T < 20°$ C).

Enzyme activity assays. Xylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) was assayed by the method of Bailey et al. (1992) using birch glucuronoxylan (Roth 7500) as substrate at 50° C and pH 5.3. Overall cellulolytic activity was assayed by the filter paper assay (FPU) and endo-1,4-β-glucanase ("cellulase", EC 3.2.1.4) using hydroxyethylcellulose (HEC) as substrate, following the standard instructions (IUPAC 1987). Soluble protein was measured by the method of Lowry et al. (1951) after precipitation with two volumes of 10% trichloracetic acid. Bovine serum albumin was used as standard.

Enzyme-aided bleaching of softwood kraft pulp. Softwood kraft pulp (kappa 23.9) was obtained from a Finnish pulp mill. The high-xylanase, low-cellulase culture filtrate concentrate from the pilot cultivation and a purified xylanase (pI 9) of *T. reesei* (Tenkanen et al. 1992) were used in the enzymatic treatments. The pulp treatments with an enzyme dosage of 500 nkat g^{-1} were carried out at 5% consistency at 45° C for 2 h in distilled water at pH 7.0. The pH of the pulp was adjusted with H_2SO_4. After enzymatic treatment the pulps were treated with 0.2% ethylenediaminetetraacetic acid (EDTA) for 1 h at 45° C to remove metals present in the pulp. Reference pulp was treated at the same pH without enzyme. The effects of the enzyme treatment were evaluated by analysing the solubilized reducing sugars by the dinitrosalicylic acid (DNS) method (Miller 1959) and also by HPLC after secondary enzymatic hydrolysis of the oligosaccharides hydrolysed in the pulp treatment (Buchert et al. 1993). Alkaline peroxide delignification of pulp [10 g dry weight (d.w.)] was carried out at 10% consistency for 60 min at 80° C. The dosages of chemicals were (per d.w. of pulp): 3% H_2O_2, 1.5% NaOH, 0.5% $MgSO_4$ and 0.2% diethylenetriaminepentaacetic acid (DTPA). After the peroxide delignification, the pulp was acidified with SO_2 and made into handsheets for the measurement of kappa number (SCAN C1:1977), brightness (ISO 2470) and viscosity (SCAN C15:1988).

Results

Simultaneous production of cellulase and xylanse by *T. reesei* VTT-D-86271 grown on cellulose-spent grain medium in the 10-l fermentor with a pH minimum control of 4.0 was used as a basis for comparison with alternative media and production conditions. The results of this cultivation are presented in Fig. 1. The culture filtrate contained both cellulolytic and xylanolytic enzymes. The production time was rather long (>100 h).

In order to test for xylanase production on media containing xylan, the fungus was first cultivated on different media in shake flasks. The carbon sources were tested at 20 g l^{-1} with 10 g l^{-1} of either SG or CSS.

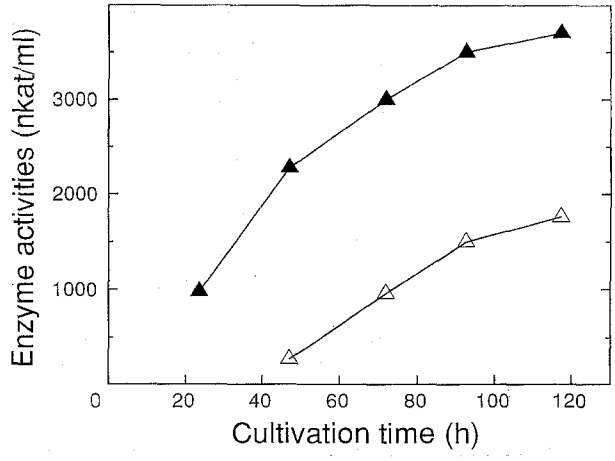

Fig. 1. Production of xylanase (▲) and cellulase (endoglucanase) using hydroxyethylcellulose as substrate (△) by *Trichoderma reesei* VTT-D-86271 on cellulose-spent grain medium in a laboratory fermenter at pH 4.0

Table 1. Production of xylanase (XYL) and cellulase using hydroxyethylcellulose (HEC) as a substrate (CEL) by *Trichoderma reesei* VTT-D-86271 on different media in shake flasks

Nutrient sources		Final pH	XYL (nkat ml^{-1})	CEL (nkat ml^{-1})
Carbon[a]	Nitrogen			
Solka floc cellulose	SG	2.9	400	420
	CSS	3.5	880	600
Beech xylan	SG	2.8	300	70
(Lenz and Schurz 1986)	CSS	3.7	700	140
Birch glucuronoxylan	SG	4.2	2100	220
(Roth 7500)	CSS	6.0	5400	50
Wheat bran	SG	5.8	2300	160
	CSS	6.3	2200	140
Birch oligosaccharides	SG	4.0	20	nd
(Stake xylan)	CSS	4.0	20	nd

The cultivation time was 7 days: SG, spent grain; CSS, corn steep solids; nd, not determined

[a] See text for details

The results of these cultivations are presented in Table 1. The best xylan source for xylanase production under these conditions was birch glucuronoxylan (Roth, product 7500). However, as this product is a fine chemical it was clearly not suitable for use on a technical scale. Although the fungus grew well on the beech xylan (visual observation), xylanase production on this carbon source was poor. By contrast, low production on the media containing steamed birch xylo-oligosaccharides was evidently due to the fact that the organism grew very poorly, if at all, on this substrate, probably due to the presence of growth inhibitors in xylan preparations obtained after steaming of wood (e.g. Buchert et al. 1990, see also Bailey and Viikari 1993). Closer examination of the results of the shake-flask cultivation (Table 1) indicated that final pH had a significant effect on xylanase production: a pH higher than 4.0 was measured in all the culture filtrates with a xylanase activity exceeding 1000 nkat ml^{-1}.

In order to investigate whether the poor production of xylanase on beech xylan medium was due only to the low pH developed on this carbon source in shake flasks (see Table 1), a series of fermenter cultivations was performed with pH control. The medium contained 30 g l^{-1} of beech xylan and 15 g l^{-1} of CSS. The pH minimum controls used were between 4.0 and 7.5. The results of these experiments demonstrated that a rather high pH (for a fungal cultivation) was essential for good production of xylanase, although growth (broth viscosity) was evidently better at pH 4.0 than at 7.0. Xylanase production in the laboratory fermentor cultivations on beech xylan at different pH values is presented in Fig. 2.

In addition to the high final level of xylanase activity, the rate of xylanase production was also much higher in the cultivations with higher pH up to 7.0. At pH 7.5 the rate of xylanase production clearly decreased, due almost certainly to slow growth of the fungus at this pH. The cultivations on xylan were characterized by rather low levels of cellulolytic activity (Table 2) and the ratio of xylanase to cellulase activities (XYL/CEL) was therefore high. The results of these epxeriments demonstrated conclusively that, despite poor results in shake-flask cultivations, the insoluble unsubstituted beech xylan was a good medium for production of essentially cellulase-free xylanase by *T. reesei*. This type of medium could also be used in technical-scale cultivations.

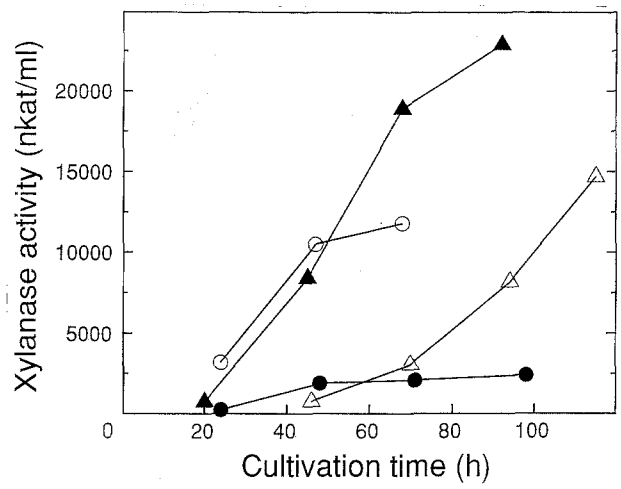

Fig. 2. Production of xylanase by *T. reesei* VTT-D-86271 on 30 g l^{-1} of beech xylan corn steep solids medium in a laboratory fermenter with different pH minimum controls: ●, pH 4.0; ○, pH 6.0; ▲, pH 7.0; △, pH 7.5

In cultivations of *T. reesei* on cellulose substrates for production of cellulases, a rather low minimum pH of between 3.0 and 4.0 has traditionally been used by most authors (e.g. Montenecourt and Eveleigh 1979; Bailey and Nevalainen 1981). When the organism was cultivated on cellulose-SG medium at pH 6.0 and 7.0, xylanase production increased considerably but production of cellulases decreased (Table 2).

In order to demonstrate the importance of xylan as an inducer of xylanase, in isolation from the effects of the secondary inducer (spent grain or corn steep solubles), two cultivations at pH 7.0 were performed in the small laboratory fermenters using 10 g l^{-1} of birch glucuronoxylan (Roth 7500) or unsubstituted insoluble

Table 2. Enzyme activities and soluble protein assayed in final culture filtrates of *T. reesei* VTT-D-86271 grown on different media and with different pH controls in a laboratory fermenter

Medium	Minimum pH	XYL (nkat ml^{-1})	CEL (nkat ml^{-1})	XYL/CEL	FPU (units ml^{-1})	Protein (g l^{-1})
Beech xylan	4.0	2400	170	14	0.8	2.0
	5.0	6300	150	42	0.6	2.2
	6.0	12000	150	80	0.4	2.8
	7.0	22900	150	150	0.7	4.2
	7.5	15000	120	125	0.3	2.6
Solka floc	4.0	3700	1800	2	9.0	13.0
	6.0	10600	1400	8	6.7	10.5
	7.0	14200	400	36	1.3	4.7

FPU, filter-paper units

beech xylan (Lenz and Schurz 1986) as carbon source with minimal salts and peptone (Mandels and Weber 1969) as the only other nutrients. The results of this experiment are presented in Fig. 3.

Xylanase production on the soluble, easily metabolizable glucuronoxylan was now rather poor, although good production was obtained with the more refractory (crystalline, insoluble) beech xylan substrate. Xylanase production on the 10 g l^{-1} beech xylan medium with minimal salts and peptone after 70 h was approximately one-third of that obtained in the same time on the same carbon source at 30 g l^{-1} using CSS as the source of organic nitrogen (cf. Figs. 2 and 3).

A cultivation was also performed in the 200-l pilot fermenter on the 30 g l^{-1} beech xylan medium with a pH minimum control of 7.0. Xylanase production in this cultivation was even slightly faster than in the laboratory fermenters (16000 nkat ml^{-1} at 50 h), probably because the final inoculum of 16 l was cultivated in a prefermenter at pH 6.5 and was therefore adapted to the high pH in the main fermentation. After cultivation the broth was separated by drum filtration with an operation time of only 10 min on the 1-m^2 filter (filtration rate 1200 l m^{-2} h^{-1}). The clarified broth (180 l) was concentrated by ultrafiltration. Although the broth was somewhat cloudy after drum filtration, due to autolysis of the fungus at the high cultivation pH, the ultrafiltration proceeded with typical dynamics for this type of culture filtrate. The permeate flux decreased steadily during the concentration, but a concentration factor of 11 was achieved with an operation time of only 4 h. The concentrate (16.2 l) contained 70% of the initial xylanase activity of the culture filtrate and less than 2% was present in the permeate. The xylanase concentrate was stabilized by precipitating with ammonium sulphate between 25 and 50% saturation, with full recovery of the xylanase activity of the liquid concentrate (Table 3).

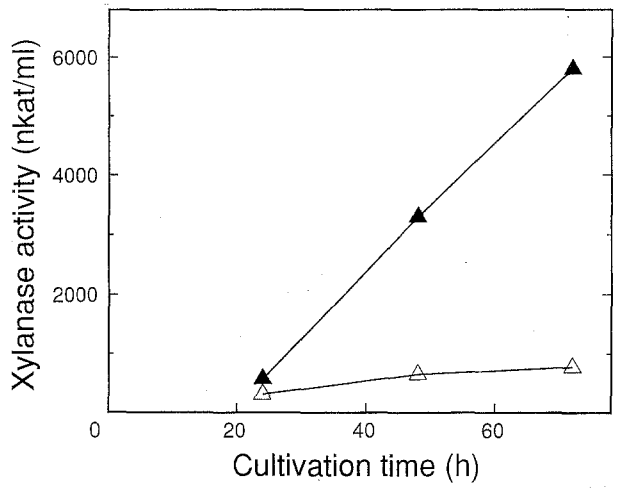

Fig. 3. Production of xylanase by *T. reesei* VTT-D-86271 on two media containing 10 g l^{-1} of birch glucuronoxylan (△) or insoluble unsubstituted beech xylan (▲) with minimal salts in a laboratory fermenter at pH 7.0

The suitability of the concentrated xylanase-rich culture filtrate produced at pH 7.0 in the pilot fermenter was tested in one-stage peroxide delignification of softwood kraft pulp. Treatment of the pulp with the

Table 3. Enzyme activities and yields obtained in the downstream processing of a pilot cultivation of *T. reesei* VTT-D-86271 on beech xylan medium at pH 7.0

Sample	Volume (l)	XYL (nkat ml^{-1})	(%)	CEL (nkat ml^{-1})	(%)	Protein (g l^{-1})	(%)
Culture filtrate	180	16000	100	160	100	2.8	100
UF concentrate	16.2	125000	70	1220	69	19.9	64
$(NH_4)_2SO_4$ concentrate[a]	1.7	1200000	70	10700	63	149.0	50

Yield percentages are based on the total activity in the culture filtrate after primary separation on the drum filter: UF, ultrafiltration

[a] Activities in the $(NH_4)_2SO_4$ slurry calculated from a 10% solution in Na-acetate buffer

Table 4. Peroxide delignification of softwood kraft pulps treated with concentrated *T. reesei* culture filtrate produced on xylan (unpurified xylanase) or with purified pI 9 xylanase of the same organism (Tenkanen et al. 1992)

Enzyme	Solubilized sugars (% of dry weight)				Kappa number	Brightness (%)	Viscosity ($dm^3\ kg^{-1}$)
	DNS	HPLC					
		Glu	Xyl	Ara			
Unpurified xylanase	0.64	<0.02	0.68	<0.02	16.0	50.8	940
pI 9 xylanase	0.58	<0.02	0.66	<0.02	16.1	50.7	960
Reference	0.10	<0.02	0.04	<0.02	18.4	48.5	960

The xylanase dosage was 500 nkat g^{-1}. Reference pulp was treated under the same conditions but without addition of enzyme. DNS, reducing sugars by the DNS method; HPLC, analysis of monomers after secondary enzymatic hydrolysis of the solubilized oligomers; Glu, glucose; Xyl, xylose; Ara, arabinose

unpurified enzyme resulted in solubilization of 0.64% of the pulp. Purified *T. reesei* pI 9 xylanase solubilized slightly less reducing sugars (0.58% of pulp d.w.). The improvement of pulp bleachability was similar with both enzyme preparations and the brightness was improved from 48.5 to 50.7–50.8. The viscosity of the pulp treated with unpurified xylanase-rich culture filtrate was only slightly lower than that of the pulp treated with purified pI 9 xylanase and that of the untreated pulp (Table 4), indicating that the xylanase produced on xylan at pH 7.0 was sufficiently free of cellulase and was suitable as such for enzymatic pulp treatments. The solubilized carbohydrates were also analysed by HPLC after secondary enzymatic hydrolysis (Buchert et al. 1993). No glucose (from cello-oligomers) was detected in the hydrolysates (Table 4).

Discussion

The results of these experiments indicate that induction of xylanases in *T. reesei* may be completely dissociated from that of cellulases. On the cellulose-based medium, production of cellulases at high pH was clearly reduced compared with production at pH 4.0, whereas xylanase production was considerably more efficient at pH 6.0–7.0. When cultivated on xylan, the fungus again produced xylanases much better at high pH (6.0–7.0) than at the more usual pH (for a fungus) of 4.0, but production of cellulases on the xylan medium was low at all the pH values tested (Table 2).

Although in the presence of a secondary inducer (SG) soluble glucuronoxylan appeared to be the best substrate for xylanase production in shake-flask experiments (Table 1), fermenter cultivations with pH control demonstrated that the less readily available, insoluble beech xylan substrate was sufficient to effect induction of high xylanase activity in the absence of any other inducers, whereas glucuronoxylan alone was not a good inducer (Fig. 3). This is in accordance with similar results obtained for production of cellulases: a more available (less crystalline) cellulase substrate is generally a poorer inducer of cellulolytic activities (Linko et al. 1977; Mes-Hartree et al. 1988).

In addition to the physical-chemical structure of the substrate, cultivation conditions may also have an effect on substrate availability. Royer and Nakas (1989), working with *T. longibrachiatum,* reported a high production of xylanases on cellulose medium at pH 7.0 (although not on xylan). These authors concluded that cultivation at the unusually high pH of 7.0 resulted in poor substrate availability, due to inefficiency of *Trichoderma* hydrolases at this pH, and therefore in growth limitation, leading to effective enzyme induction. The present results with *T. reesei* are in good agreement with this hypothesis, although with this organism xylan was also an effective inducer of xylanases. Royer and Nakas (1989) used only soluble (oat spelt) xylan, with results similar to those obtained with soluble glucuronoxylan in the fermenter in this work (Fig. 3). It is possible that the strain of *T. longibrachiatum* studied by Royer and Nakas (1989) would also produce xylanases well at high pH on the insoluble beech xylan used in this work.

The effect of pH on production of xylanases by *T. reesei* was totally different from that recently reported by these authors when working with *Aspergillus fumigatus* (Bailey and Viikari 1993), for which a pH below 3.0 was found to be essential for efficient production of xylanase on the same beech xylan substrate as that used in this work. It is therefore obvious that enzyme production results obtained for one fungal organism do not necessarily apply to another.

The results of the pilot cultivation and the subsequent downstream processing demonstrated that the production process developed in this work would be readily applicable in industrial-scale production. Production times were short and separation and concentration of the xylanase by standard procedures presented no technical problems. Partial purification of the xylanase for removal of cellulase activity was not necessary when the enzyme was used for bleaching of kraft pulp (Table 4). The xylanase-rich culture filtrate concentrate was suitable as such for enzyme-aided bleaching of softwood kraft pulp, giving an effect similar to that of the purified pI 9 xylanase with respect to both hydrolysis of pulp carbohydrates and improvement of the bleachability.

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