World J Microbiol Biotechnol (2008) 24:605–612
DOI 10.1007/s11274-007-9516-2

ORIGINAL PAPER

Production, purification and characterization of *Bacillus* sp. GRE7 xylanase and its application in eucalyptus Kraft pulp biobleaching

Jeyagowri Kiddinamoorthy · Alfredo J. Anceno · Gulelat D. Haki · Sudip K. Rakshit

Received: 1 September 2006 / Accepted: 18 January 2007 / Published online: 16 August 2007

Abstract Production of extracellular xylanase from *Bacillus* sp. GRE7 using a bench-top bioreactor and solid-state fermentation (SSF) was attempted. SSF using wheat bran as substrate and submerged cultivation using oat-spelt xylan as substrate resulted in an enzyme productivity of 3,950 IU g^{-1} bran and 180 IU ml^{-1}, respectively. The purified enzyme had an apparent molecular weight of 42 kDa and showed optimum activity at 70°C and pH 7. The enzyme was stable at 60–80°C at pH 7 and pH 5–11 at 37°C. Metal ions Mn^{2+} and Co^{2+} increased activity by twofold, while Cu^{2+} and Fe^{2+} reduced activity by fivefold as compared to the control. At 60°C and pH 6, the K_m for oat-spelt xylan was 2.23 mg ml^{-1} and V_{max} was 296.8 IU mg^{-1} protein. In the enzymatic prebleaching of eucalyptus Kraft pulp, the release of chromophores, formation of reducing sugars and brightness was higher while the Kappa number was lower than the control with increased enzyme dosage at 30% reduction of the original chlorine dioxide usage. The thermostability, alkali-tolerance, negligible presence of cellulolytic activity, ability to improve brightness and capacity to reduce chlorine dioxide usage demonstrates the high potential of the enzyme for application in the biobleaching of Kraft pulp.

Keywords *Bacillus* sp. GRE7 · Xylanase · Biobleaching · Kraft pulp

J. Kiddinamoorthy · A. J. Anceno · S. K. Rakshit (✉)
School of Environment, Resources and Development, Asian Institute of Technology (AIT), Klong Luang, Pathumthani 12120, Thailand
e-mail: rakshit@ait.ac.th

G. D. Haki
Awassa College of Agriculture, Debub University, Awassa, Ethiopia

Introduction

Xylan, a major constituent of hemicellulose, is composed of $\beta-1$, 4-linked xylopyranosyl residues which can be substituted with arabinosyl and methylglucuronyl side-chains. Xylanases (endo−1, 4-β-D-xylan xylanohydrolase; E.C. 3.2.1.8) are a group of enzymes that hydrolyse xylan backbone into small oligomers. A wide variety of micro-organisms, mainly bacteria and fungi, are known to produce xylan-degrading enzymes. In recent years, important biotechnological applications of xylanases include various processes in the food, animal feed and pulp and paper industries (Beg et al. 2001; Techapun et al. 2003).

Large amounts of xylanases are required in Kraft pulp prebleaching in the pulp and paper industry, and this is so far the biggest potential application of xylanolytic enzymes (Viikari et al. 1986; Kulkarni et al. 1999). There is limited understanding on the mechanism by which enzymatic prebleaching is facilitated. It was proposed however that xylanases depolymerize hemicellulose precipitated on the surface of the fibre that opens up the pulp structure and allows access of chlorine and other chemicals employed in subsequent treatment stages (Paice et al. 1988). This in turn facilitates pulp delignification and bleach boosting, imparting brightness to the paper. Enzymatic prebleaching option is said to be ecofriendly (Beg et al. 2001; Sá-Pereira et al. 2002), as it reduces the amount of environmentally hazardous and toxic organochlorines in effluents from the conventional chemical treatment sequence employing chorine dioxide and alkaline extraction.

While enzymatic prebleaching requires a thermostable and alkalitolerant enzyme, only a few xylanases are reportedly active and stable at both alkaline pH and elevated temperatures (Nakamura et al. 1993; Subramaniyan et al. 2001). Most of the commercially available xylanases

showing stability at alkaline conditions are only optimally active at 30–60°C (Dhillon et al. 2000; Techapun et al. 2003). On the other hand, xylanases produced by members of the genus *Bacillus* exhibit a reasonable thermostability ranging from acid to alkaline pH (Subramaniyan and Prema 2000; Haki and Rakshit 2003). Continuing interest in *Bacillus* sp. xylanases is also evident in the recent papers, among others, handling several aspects from enzyme production to application (Avcioglu et al. 2005; Heck et al. 2005; Sapre et al. 2005; Virupakshi et al. 2005; Battan et al. 2006; Choudhury et al. 2006; etc.). The present paper reports the exceptionally high production, purification, characterization and application in Kraft pulp biobleaching of an extracellular xylanase produced by *Bacillus* sp. GRE7 isolated from Ethiopian hot spring soil.

Materials and methods

Bacterial strain and culture conditions

Bacillus sp. GRE7, isolated from an Ethiopian hyperthermal spring soil (Haki 2003), was identified using standard biochemical tests (Claus and Berkely 1986) and API50CH (bioMérieux, Marcy-l'Etoile, France) carbohydrate metabolism profiling at the Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, Thailand. The bacterium was maintained and routinely subcultured on a basal medium containing (w v^{-1}) 0.5% peptone, 0.1% NaCl, 0.2% K_2HPO_4, 0.01% $CaCl_2$, 0.01% $MgSO_4$, 0.1% yeast extract, 1.5% bacteriological agar and 0.5% oat-spelt xylan (Sigma, Munich, Germany). This strain grew very well at 50–55°C. Stock cultures were stored at 4°C.

Production of enzyme

Xylanase production by submerged fermentation (SmF) was carried out in 1-l Biostat Q bioreactors (B. Braun, Germany) using the basal liquid medium containing (w v^{-1}) 0.5 or 1% oat-spelt xylan with the following culture condition: volume, 750 ml; pH 7–7.5; temperature, 55°C; agitation, 200 rev. min^{-1}; aeration, 0.5 l min^{-1}; inoculum (20-h old) size, 10% (v v^{-1}). To monitor the time course of bacterial growth and enzyme production, samples were collected at fixed time intervals and cells were separated by centrifugation at 12,000× *g* for 10 min at 4°C. The cell-free culture supernatant was used as crude enzyme source for xylanase assay.

Enzyme production by solid-state fermentation (SSF) was carried out according to the procedure suggested by Archana and Satyanarayana (1997). The same medium and culture conditions were employed with an incubation temperature of 55°C. Flasks containing bacterial bran were removed at fixed time intervals. Enzyme from contents was extracted twice with 20 mM sodium phosphate buffer at pH 7 (10 ml g^{-1} wheat bran) and squeezed through a wet muslin cloth. The enzyme extract was centrifuged at 15,000× *g* for 30 min and the clear supernatant was used in xylanase assay.

Purification of enzyme

All steps during purification were performed at 4°C. In order to obtain the crude extracellular xylanase, culture broth from 1-l bioreactors, 1% oat-spelt xylan cultures (SmF), were centrifuged at 10,000× *g* for 30 min. The cell-free culture supernatant was brought to 40–80% ammonium sulphate (AMS) saturation, left for sedimentation and centrifuged at 10,000× *g* for 1 h. Protein precipitates were harvested and dissolved in sodium phosphate buffer (20 mM, pH 7). The solution was dialysed against the same buffer overnight and lyophilized. The crude enzyme was dissolved in a minimum volume of sodium phosphate buffer (20 mM, pH 7), loaded onto a DEAE-cellulose column (30 × 2 cm^2) equilibrated with the same buffer. Proteins were eluted with discontinuous NaCl gradient (0–5 M) in the same buffer at 30 ml h^{-1}. Active fractions were pooled, lyophilized, dissolved in sodium phosphate buffer (20 mM, pH 7) and applied onto a Sephadex G-75 column (50 × 1 cm^2) equilibrated with the same buffer and eluted at 18 ml h^{-1}. Active fractions were pooled, dialysed in sodium phosphate buffer (20 mM, pH 7) and used for further experiments.

Enzyme and protein assays

Xylanolytic and cellulolytic activities were determined by measuring the amount of reducing sugar liberated using dinitrosalicylic acid (DNS) (Miller 1959). Xylanase activity was assayed by adding 0.1 ml of an enzyme preparation to 0.4 ml of a 0.5% oat-spelt xylan suspension in 20 mM sodium phosphate buffer (pH 7). Assay for saccharifying cellulase was carried out according to Ghose (1987) with modification. A Whatman no. 1 filter paper strip (1 × 6 cm ≈ 50 mg) was added to a mixture of 0.1 ml of an enzyme preparation and 0.4 ml 20 mM sodium phosphate buffer (pH 7). The enzyme reaction was carried out at 60 and 58°C for 15 and 60 min, respectively, and terminated by adding 0.5 ml DNS solution. Reaction mixtures were boiled for 10 min, added with 10 ml distilled water and cooled to stabilize the colour. Absorbance was measured at 540 nm against blank. One unit (IU) of xylanase or cellulase activity is defined as the amount of enzyme that

catalyses the release of 1 μmol reducing sugar as xylose or glucose equivalent per min under the conditions described above.

The protein concentration in chromatographic eluates was determined by measuring the absorbance at 280 nm. Total soluble protein content at the end of each purification step was determined by the Bradford method using bovine serum albumin as standard.

Electrophoresis and zymography

The predominant xylanase of *Bacillus* sp. GRE7 was subjected to SDS-PAGE (Laemmli 1970) using 10% acrylamide gel containing 0.1% xylan. Electrophoresis was carried out using the Mini-Protean II system (Bio-Rad, Hercules, CA, USA) and protein bands were stained with Coomassie brilliant blue R-250. Protein molecular weight standards used were bovine serum albumin (66.2 kDa), chicken ovalbumin (45 kDa), horseradish peroxidase (40 kDa), cytochrome *c* (12.5 kDa) and bovine insulin chain B (3.4 kDa).

A duplicate lane was subjected to activity staining according to Tseng et al. (2002) with slight modification. After electrophoresis, the gel was washed twice for 30 min in 0.1 M Tris–HCl buffer (pH 7) containing 25% isopropanol to remove SDS. The gel was incubated in the same buffer without isopropanol for 30 min at 50°C. The gel was soaked in 0.1% Congo red solution for 15 min at 30°C and destained in 1 M NaCl. The xylanase band was revealed against the dark background by treatment with 0.5% acetic acid solution.

Biochemical characterization

The optimum pH for xylanase activity was determined by using 50 mM of the following buffer systems: acetate buffer (pH 4–5), phosphate buffer (pH 6–7), Tris–HCl buffer (pH 8–9), glycine-NaOH buffer (pH 10) and Na_2HPO_4–NaOH (pH 11–12). The enzyme was incubated with 0.5% oat-spelt xylan in different buffers at 60°C for 15 min and analysed for reducing sugar. The optimum temperature for xylanase activity was determined by incubating the reaction mixture at different temperatures ranging from 40 to 100°C at pH 7. Stability at different pH was determined by incubating the enzyme in the same buffer systems for 30 min at 37°C. Thermostability was determined by incubating the enzyme at different temperatures in 50 mM phosphate buffer (pH 7). Residual enzyme activity was measured by the standard assay. The effect of the presence of different concentrations of metal ions (1–10 mM) on xylanase activity was measured under standard assay conditions. K_m and V_{max} for oat-spelt xylan (2.5–12.5 mg ml^{-1}) at 60°C and pH 6 was determined by a Lineweaver–Burk plot.

Application of xylanase in Kraft pulp bleaching and pulp analyses

Eucalyptus Kraft pulp samples (12 g oven dried) were treated with *Bacillus* sp. GRE7 xylanase at 1–10 IU g^{-1} dry pulp in plastic bags with intermittent kneading. Pulp consistency of 10% (w v^{-1}) was achieved by adding 20 mM phosphate buffer (pH 7). Samples were incubated for 3 h at 60°C. Control samples were subjected to the same treatment conditions without enzyme treatment. Pulp slurries were filtered after incubation and filtrates were analysed for the release of chromophoric material by spectrophotometry (280 and 456 nm) and reducing sugars (Miller 1959). Xylanase-treated and untreated pulp samples were chemically bleached using the DED (chlorine dioxide [D_1]−alkali extraction [E]−chlorine dioxide [D_2]) treatment sequence according to Madlala et al. (2001). Usage of chlorine dioxide (ClO_2) was reduced by 30%, corresponding to a decrease in the consumption of the bleaching agent from 2.63 to 1.84% (w w^{-1}) in D_1 and 1.31 to 0.92% (w w^{-1}) in D_2. After enzyme treatment, pulps were analysed for Kappa number according to SCAN-C 1:77. After chemical treatment, pulps were prepared as handsheets according to SCAN-C M11 for brightness determination according to Technical Association of Pulp and Paper Industry (TAPPI) protocol T-452 om-92.

Results and discussion

The time course of xylanase production by *Bacillus* sp. GRE7 was monitored during SmF with oat-spelt xylan in a 1-l reactor (Fig. 1a). Highest xylanase activity (60–80 IU ml^{-1}) using 0.5% oat-spelt xylan occurred after 5–18 h of fermentation, in agreement with a previous report on higher xylanase production by *Bacillus subtilis* after 10–15 h of fermentation at 55°C in 2-l reactors (Sá-Pereira et al. 2002). Optical density readings at 600 nm indicated a gradual increase of cell mass up to the stationary phase after 5 h of fermentation while pH of the culture media was 7.3–7.7 during 24-h cultivation (data not shown). A similar experiment using 1% oat-spelt xylan (Fig. 1a) gave an approximately twofold increase in enzyme activity (100–170 IU ml^{-1}) after 18 h with values for culture parameters in ranges comparable to that of the experiment using 0.5% xylan (data not shown). The highest enzyme yield value corresponds to a specific productivity of 17,000 IU g^{-1} oat-spelt xylan. As studies on xylanase

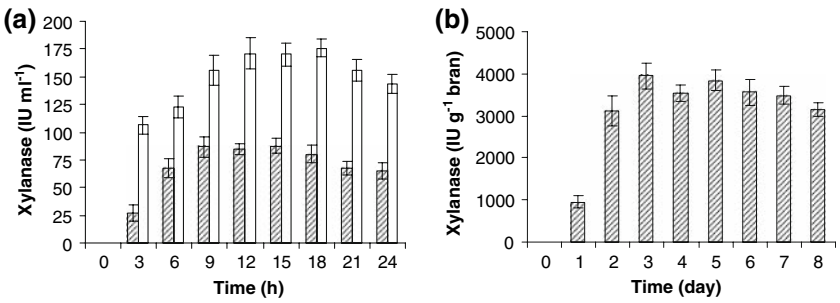

Fig. 1 Time course of xylanase production by *Bacillus* sp. GRE7. (**a**) Submerged cultivation in 1-l bioreactors using 0.5 and 1% w v^{-1} oat-spelt xylan (*shaded* and *unshaded* columns, respectively); (**b**) Solid-state fermentation using 40% w v^{-1} wheat bran

production by different microorganisms usually use 0.5–1% xylan (Techapun et al. 2003), higher xylan concentrations were not tested further.

The use of SSF, on the other hand, offers several advantages over SmF. These include simplicity of the fermentation media, elimination of expensive control systems and complex machinery, greater product yield with lesser fermentation vessel volume, easier control of contamination due to low moisture level in the system, lower downstream processing cost, and lower overall capital requirement (Archana and Satyanarayana 1997). While fungal contamination is most likely in mesophilic systems, many fungi cannot grow at 50 °C at which the present SSF is carried out for *Bacillus* sp. GRE7 and in that sense contamination is better controlled under thermophilic SSF. In the case of SSF employing alkaliphic bacilli (Battan et al. 2006), the unfavourable pH will also minimize contamination. As regards downstream processing, it is straightforward that since a lower solvent volume is required for product recovery from a lower fermentation volume, SSF yields high xylanase titers which in turn lead to fewer purification steps. The energy demand is lower, since no agitation and aeration is needed, only extended temperature maintenance. Moreover, the use of simple media and cheap lignocellulosic substrates, fewer downstream processing steps due to less volume of liquid required for product recovery, and elimination of expensive machinery and sophisticated control systems in SSF will far off-set the cost of energy required in thermophilic SSF for an extended time.

Although a large number of fungal species are known to grow well on moist substrates and are thus used in many SSF studies, several bacterial strains, mostly *Bacillus* sp., are reported to have successfully produced industrially significant enzymes via SSF (Lonsane and Ramesh 1990; Pandey 1992; Archana and Satyanarayana 1997; Gessesse and Mamo 1999; Virupakshi et al. 2005; Battan et al. 2006; etc.). In the present study, *Bacillus* sp. GRE 7 was employed in xylanase production via SSF using wheat bran as substrate (Fig. 1b). A relatively low level of xylanase productivity was observed after few hours of fermentation (data not shown) but steadily increased and peaked after 3 days. Productivity remained almost stable until 7 days but did not increase further with prolonged incubation. The maximum value of xylanase productivity of *Bacillus* sp. GRE7 on wheat bran obtained via SSF was ~3,950 IU g^{-1} bran. This was among the highest values obtained by similar studies made earlier and closely comparable to that of *Bacillus* sp. JB-99 (3,644 IU g^{-1}) SSF-cultivated on rice bran (Virupakshi et al. 2005) and *Bacillus pumilus* ASH (5,300 IU g^{-1}) SSF-cultivated on rice bran (Battan et al. 2006). A very minimal cellulase activity of 0.57 IU ml^{-1} of culture supernatant and 9.85 IU g^{-1} bran from SmF and SSF was measured, respectively.

Table 1 summarizes the purification stages for *Bacillus* sp. GRE7 xylanase. AMS (40–80%) precipitation resulted in a 71% recovery of initial xylanase activity in the culture supernatant. Fractions eluted during ion-exchange (DEAE-cellulose) chromatography gave two active xylanase components (data not shown). Multiplicity of high- and low-molecular weight xylanases observed in *Bacillus* spp. was explained to be due to distinct coding genes (Wong et al. 1988), differential glycosylation at post-translation or proteolysis (Bernier et al. 1983). The major and minor components constituted about 48% and <3% of the total xylanase activity, respectively, but only the major

Table 1 Summary for *Bacillus* sp. GRE7 xylanase purification

Purification steps	Total activity (IU)	Total protein (mg)	Specific activity (IU mg^{-1})	Purification (fold)	Recovery (%)
Culture supernatant	33,163	680	48.8	1.0	100
AMS precipitation	23,645	124	191.1	3.9	71
DEAE-cellulose	15,957	27.4	582.9	11.9	48
Sephadex G-75	8,878	6.4	1,392.6	28.5	27

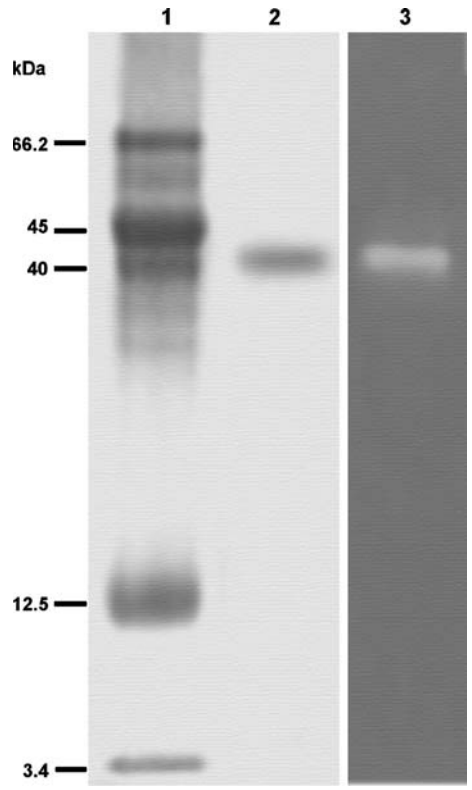

Fig. 2 SDS-PAGE of purified *Bacillus* sp. GRE7 xylanase. Lane 1, molecular weight markers; Lane 2, purified xylanase; Lane 3, zymogram visualizing activity

component was purified further by gel filtration (Sephadex G-75). The latter procedure yielded an overall xylanase recovery of 27% and 28.5-fold purification. SDS-PAGE revealed a single band, indicating that the enzyme was purified to apparent homogeneity, while zymography showed a corresponding sharp activity band corresponding to a 42-kDa protein estimated from the migration of molecular weight standards after electrophoresis (Fig. 2). No cellulase activity was detected in the purified enzyme from SmF. Although a highly purified enzyme is requisite to characterization and structural studies, it may not be economical and thus unnecessary in some applications like biobleaching. It has been shown that a crude enzyme preparation gives hydrolysis product profile similar to a purified counterpart (Dhillon et al. 2000). Moreover, different xylanase isozymes, present in one organism, with dissimilar mode of action or substrate specificities (Elegir et al. 1994) could synergistically function in xylan hydrolysis (He et al. 1994).

In an activity assay using different pH buffers, peak activity of *Bacillus* sp. GRE7 xylanase was observed at pH 7 and more than 60% activity was retained at pH 6 and 8 (Fig. 3a). The enzyme also showed stability at pH 6–11 in an assay following pre-incubation at various pH (Fig. 3b). More than 80 and 60% activity were retained at pH 6–8 and pH 9–11, respectively. Enzyme activity was also measured at different temperatures and was found optimum at 70°C (Fig. 3c). The enzyme showed thermal stability in a broad range of temperatures (Fig. 3d). Half-life values of 20 and 10 min were obtained at temperatures 80 and 90°C, respectively. More than 70% activity was retained after 135-min incubation at 60 and 70°C. Table 2 shows the effect of metal ions on xylanase activity. Positive modulatory effect on enzyme activity was obtained with Mn^{2+}

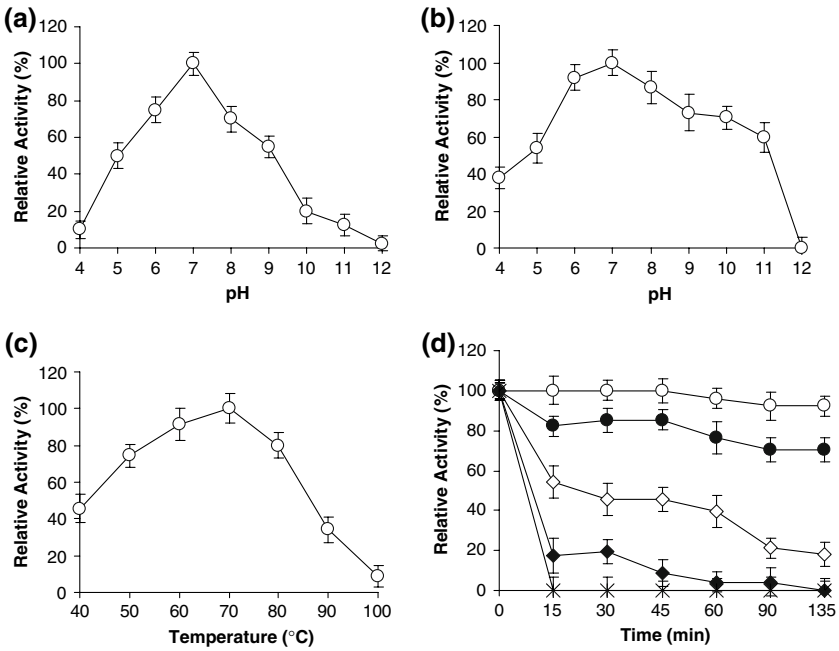

Fig. 3 Determination of optimum characteristics of *Bacillus* sp. GRE7. (**a**) Optimum pH; (**b**) pH stability; (**c**) Optimum temperature; and (**d**) Thermostability at 60°C (●), 70°C (◯), 80°C (◇), 90°C (◆), 100°C (*asterisks*)

Table 2 Residual activity of *Bacillus* sp. GRE7 xylanase at different metal ion concentrations

Cation	Concentration		
	1 mM	5 mM	10 mM
None[a]	100	100	100
$CaCl_2$	100	123	100
$CdCl_2$	75	113	109
$CoCl_2$	173	181	155
$CuSO_4$	38	31	25
$FeCl_2$	97	55	14
KCl	107	71	38
$MgCl_2$	130	49	24
$MnCl_2$	177	184	228
NaCl	83	71	45
$ZnCl_2$	38	106	81

[a] Activity in the absence of metal ions; 10 IU of enzyme was used per assay

followed by Co^{2+} while the presence of Cu^{2+}, Fe^{2+} and Na^{+} inhibited the enzyme. The rest of the metal ions caused a range of concentration dependent stimulatory effect on enzyme activity. Albeit most of the reported *Bacillus* sp. xylanases are strongly inhibited by Mn^{2+}, the present finding rather agrees with those of Bataillon et al. (2000) and Sá-Pereira et al. (2002). *Bacillus* sp. GRE7 xylanase could be a metalloprotein and metal ions that positively stimulated its activity may be employed as catalysts in trace amounts during application. Moreover, the present data can be used to determine the amount of enzyme required for different pulp streams with varying metal levels due to the different quality of water used and available. The enzyme obeyed Michaelis-Menten kinetics with an apparent K_m of 2.23 mg ml^{-1} and V_{max} of 7.42 μmol min^{-1} ml^{-1} (296.8 μmol min^{-1} mg^{-1} protein) as determined by a Lineweaver–Burk plot of the initial reaction rates at different concentrations of oat-spelt xylan (Fig. 4). The present results are consistent with the generally reported values for *Bacillus* sp. (Beg et al. 2001).

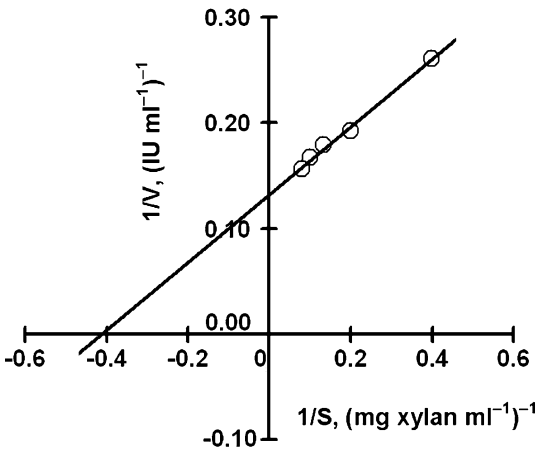

Fig. 4 Lineweaver–Burke plot of initial velocity data for *Bacillus* sp. GRE7 xylanase on oat-spelt xylan (2.5–12.5 mg ml^{-1}) measured at 60°C and pH 6

Treatment of Kraft pulp with xylanases improves lignin extractability (Kappa number reduction). It also releases different amounts of reducing sugars (xylooligosaccharides) and chromophoric (u.v.-absorbing) products such as hexeneuronic acid (HexA), formed from the 4-*O*-methyl-D-glucuronic acid component of xylan during Kraft pulping. While the release of reducing sugars only measures xylanase activity, it is the release of chromophoric materials that correlates significantly with pulp bleachability (Patel et al. 1993). Xylanase prebleaching at high temperatures improves important pulp quality indicators such as Kappa number (degree of delignification) and brightness (bleaching effectiveness). In the present study, released chromophores and reducing sugars from eucalyptus Kraft pulp were measured after xylanase pretreatment. Kappa number and brightness was also measured following ClO_2–NaOH-based DED bleaching sequence of the xylanase prebleached pulp. The results are shown in Table 3. A marked increase in the release of chromophores and reducing sugars resulted from increments of xylanase dosage (1–10 IU g^{-1} pulp) with an observed positive general correlation between the values of these parameters. A xylanase charge of 10 IU g^{-1} pulp brought the highest Kappa number reduction and

Table 3 Effect of *Bacillus* sp. GRE7 xylanase pretreatment on the release of chromophoric material, reducing sugars, Kappa number and brightness of eucalyptus Kraft pulp

Enzyme charge (IU g^{-1} pulp)	Chromophore release		Reducing sugars (mg g^{-1} pulp)	Kappa number[a]	Decrease in Kappa number (points)	Brightness[a] (% ISO)	Gain in brightness (points)
	(A_{280})	(A_{465})					
0	0.568 ± 0.018	0.016 ± 0.005	0.28 ± 0.09	2.16 ± 0.20	0	85.53 ± 1.02	0.00
1	1.015 ± 0.012	0.037 ± 0.007	0.84 ± 0.12	1.79 ± 0.14	0.37	86.71 ± 0.98	1.08
5	1.030 ± 0.009	0.040 ± 0.004	1.12 ± 0.10	1.28 ± 0.11	0.88	87.69 ± 1.13	2.16
10	1.116 ± 0.015	0.048 ± 0.009	1.39 ± 0.17	1.15 ± 0.09	1.01	88.22 ± 1.21	2.69

[a] Parameters measured after DED bleaching sequence at 30% total reduction of the original chlorine dioxide usage

brightness gain of 1.01 and 2.7 points, respectively. This implies savings in ClO_2 consumption of up to 30% (4.5 kg t^{-1}) with reasonable quality Kraft pulp. In a previous study using 10 IU g^{-1} commercial Xylanase P, the control level of brightness was retained at 23.3, 30 and 16.7% ClO_2 reduction for bagasse, soda-aq, and Kraft pulps, respectively (Madlala et al. 2001). In another study, the use of commercial xylanases Novozyme 473 and VAI-Xylanase increased brightness of Kraft pulp by 2.5 points at 31% ClO_2 reduction (Bajpai et al. 1994). Our results therefore suggest that the present enzyme could sufficiently match the performance of commercially available xylanase preparations. As the extent of ClO_2 usage during chemical bleaching was found to depend on the type of pulp and enzyme used (Madlala et al. 2001), further studies on prebleaching different pulp types with *Bacillus* sp. GRE 7 xylanase will be carried out.

Acknowledgements The Dutch Government awarded a scholarship to the first author during the course of this investigation. The Pulp and Paper Technology Laboratory at the AIT (Thailand) provided paper pulp samples and accommodated pulp analyses.

References

Archana A, Satyanarayana T (1997) Xylanase production by thermophilic *Bacillus licheniforms* A99 in solid-state fermentation. Enzyme Microb Technol 21:12–17

Avcioglu B, Eyupoglu B, Bakir U (2005) Production and characterization of xylanases of a *Bacillus* strain isolated from soil. World J Microbiol Biotechnol 21:65–68

Bajpai P, Bhardwaj NK, Bajpai PK (1994) The impact of xylanases on bleaching of eucalyptus Kraft pulp. J Biotechnol 38:1–6

Bataillon M, Cardinali APN, Castillon N, Duchiron F (2000) Purification and characterization of a moderately thermostable xylanase from *Bacillus* sp. strain SPS-0. Enzyme Microb Technol 26:187–192

Battan B, Sharma J, Kuhad RC (2006) High-level xylanase production by alkaliphilic *Bacillus pumilus* ASH under solid-state fermentation. World J Microbiol Biotechnol 22:1281–1287

Beg QK, Kapoor M, Mahajan L, Hoondal GS (2001) Microbial xylanases and their industrial applications: a review. Appl Microbiol Biotechnol 56:326–338

Bernier R, Driguez JR, Desrochers M (1983) Molecular cloning of *Bacillus subtilis* xylanase gene in *Escherichia coli*. Gene 26:59–65

Choudhury B, Chauhan S, Singh SN, Ghosh P (2006) Production of Xylanase of *Bacillus coagulans* and its bleaching potential. World J Microbiol Biotechnol 22:283–288

Claus D, Berkely RCW (1986) Genus *Bacillus* Cohn 1872. In: Sneath PHA (ed) Bergey's manual of systematic bacteriology. Williams and Wilkins, Baltimore, MD, USA, pp 1105–1139

Dhillon A, Gupta JK, Khanna S (2000) Enhanced production, purification and characterization of a novel cellulase-poor, thermostable, alkalitolerant xylanase from *Bacillus circulans* AB16. Process Biochem 35:849–856

Elegir G, Szakács G, Jeffries TW (1994) Purification, characterization and substrate specificities of multiple xylanases from *Streptomyces* sp. strain B-12-2. Appl Environ Microbiol 60:2609–2615

Gessesse A, Mamo G (1999) High-level xylanase production by an alkaliphilic *Bacillus* sp. by using solid-state fermentation. Enzyme Microb Technol 25:68–72

Ghose TK (1987) Measurement of cellulase activities. Pure Appl Chem 59:257–268

Haki GD (2003) Thermostable α-amylases from hyperthermal springs of Ethiopia: isolation, purification, characterization and application. Ph.D. dissertation PH-03-6, Asian Institute of Technology, Klong Luang, Pathumthani, Thailand

Haki GD, Rakshit SK (2003) Developments in industrially important thermostable enzymes: a review. Bioresour Technol 89:17–34

He L, Bickerstaff GF, Paterson A, Buswell JA (1994) Evaluation of catalytic activity and synergism between two xylanase isoenzymes in enzymatic hydrolysis of two separate xylans in different states of solubility. Enzyme Microb Technol 16:696–702

Heck JX, Flôres SH, Hertz PF, Ayub MAZ (2005) Optimization of cellulase-free xylanase activity produced by *Bacillus coagulans* BL69 in solid-state cultivation. Process Biochem 40:107–112

Kulkarni N, Abhay S, Rao M (1999) Molecular and biotechnological aspects of xylanases. FEMS Microbiol Rev 23:411–456

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227:680–685

Lonsane BK, Ramesh MV (1990) Production of bacterial thermostable α-amylase by solid-state fermentation: a potential tool for achieving economy in enzyme production and starch hydrolysis. Adv Appl Microbiol 35:1–56

Madlala AM, Bissoon S, Singh S, Christov L (2001) Xylanase-induced reduction of chlorine dioxide consumption during elemental chlorine-free bleaching of different pulp types. Biotechnol Lett 23:345–361

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428

Nakamura S, Wakabayashi K, Nakai R, Ano R, Horikoshi K (1993) Purification and some properties of an alkaline xylanase from alkaliphilic *Bacillus* sp. strain 41M-1. Appl Environ Microbiol 59:2311–2316

Paice MG, Bernier R Jr, Jurasek L (1988) Viscosity enhancing bleaching of hardwood Kraft pulp with xylanase from a cloned gene. Biotechnol Bioeng 32:235–239

Pandey A (1992) Recent process developments in solid-state fermentation. Process Biochem 27:109–117

Patel RN, Grabski AC, Jeffries TW (1993) Chromophore release from Kraft pulp by purified *Streptomyces roseiscleroticus* xylanases. Appl Microbiol Biotechnol 39:405–412

Sá-Pereira P, Mesquita A, Duarte JC, Barro MRA, Costa-Ferreira M (2002) Rapid production of thermostable cellulase-free xylanase by a strain of *Bacillus subtilis* and its properties. Enzyme Microb Technol 30:924–933

Sapre MP, Jha H, Patil MB (2005) Purification and characterization of a thermoalkalophilic xylanase from *Bacillus* sp. World J Microbiol Biotechnol 21:649–654

Subramaniyan S, Prema GS (2000) Cellulase-free xylanases from *Bacillus* and other microorganisms. FEMS Microbiol Lett 183:1–7

Subramaniyan S, Sandhia GS, Prema P (2001) Control of xylanase production without protease activity in *Bacillus* sp. by selection of nitrogen source. Biotechnol Lett 23:369–371

Techapun C, Poosaran N, Masanori W, Sasaki K (2003) Thermostable and alkaline-tolerant microbial cellulose-free xylanases produced from agricultural wastes and the properties required for use in pulp bleaching bioprocesses: a review. Process Biochem 38:1327–1340

Tseng M-J, Yap M-N, Ratanakhanokchai K, Kyu KL, Chen ST (2002) Purification and characterization of two cellulose-free

xylanase from an alkaliphilic *Bacillus firmus*. Enzyme Microb Technol 30:590–595

Viikari L, Ranua M, Kantelinen A, Sundquist J, Linko M (1986) Bleaching with enzymes. In: Proceedings of the 3rd international conference of biotechnology on pulp and paper industry. Stockholm, 16–19 June, pp 67–69

Virupakshi S, Babu KG, Gaikwad SR, Naik GK (2005) Production of a xylanolytic enzyme by a thermoalkalophilic *Bacillus* sp. JB-99 in solid-state fermentation. Process Biochem 40:431–435

Wong KKL, Tan LUL, Saddler JN (1988) Multiplicity of β-1-4-xylanase in microorganisms: functions and applications. Microbiol Rev 52:305–317