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Xylan-binding xylanase Xyl30 from *Streptomyces avermitilis*: cloning, characterization, and overproduction in solid-state fermentation

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Summary. A DNA fragment from the lignocellulolytic actinomycete *Streptomyces avermitilis* CECT 3339 was cloned using a DNA probe from the xylanase gene *xysA* of *Streptomyces halstedii.* The nucleotide sequence analysis revealed two potential ORFs, *xyl30* and *hd30*, encoding a deduced multimodular F/10 xylanase with a binding domain and a secreted glycoxyl hydrolase, respectively. In *Streptomyces lividans* carrying the subcloned DNA fragment, two xylanase activity bands with estimated molecular masses of 42.8 and 35 kDa (named Xyl30 forms "h" and "l", respectively), were detected by zymograms and SDS-PAGE. The two xylanases had identical N-terminal sequences, suggesting that Xyl30 "l" derived from Xyl30 "h" by C-terminal processing in the culture supernatant. No transcripts of *hd30* were detected by RT-PCR. Characterization of the partially purified Xyl30 "h" confirmed the presence of a modular endoxylanase containing a xylan-binding domain, which after processing in the culture supernatant loses the aforementioned domain and thus its capacity to bind xylan (Xyl30 "l"). Xyl30 "h" achieved maximal activity at pH 7.5 and 60°C, retaining more than 50% of its activity from pH 3 to 9 and more than 40% after a 1-h incubation at 70ºC. Moreover, in the recombinant host strain up to 400 U xylanase/g medium (dry weight) was produced in solid-state fermentation (SSF) using cereal bran as substrate. The high production yields of this enzyme and its biochemical features make it a good candidate for use in industrial applications. [**Int Microbiol** 2008; 11(2):133-141]

Key words: *Streptomyces avermitilis* · xylanase · xylan-binding module · heterologous production · solid-state fermentation (SSF)

Introduction

Wood and other lignocellulosic materials are formed by three main polymeric constituents: cellulose, lignin, and hemicelluloses [20]. Hemicellulose is the second most abundant plant fraction available naturally. Xylan is the major hemicellulose-type polysaccharide, accounting for 30% of the cell wall content of annual plants, 15–30% of hardwoods, and 7–10% of softwoods [24]. Basically, the xylan structure of terrestrial plants consists of D-xylopiranose units linked by β-1,4 bonds; depending on the source, the backbone may contain a varying degree of glucuronosyl, 4-*o*-methyl-D-glucuronopyranosyl, α-l-arabinofuranosyl, acetyl, feruloyl, and/or *p*-coumaroyl substituents [21].

Xylanases are endo-(1,4)-β-xylanases (EC 3.2.1.8) that depolymerize the xylan backbone by cleaving the β-(1,4) glycosidic bonds between D-xylose residues in the main chain to produce short xylooligosaccharides. Xylanases are

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found in a cornucopia of saprophytic and phytopathogenic organisms, such as bacteria, mycorrhizic fungi, and some yeasts. These enzymes can be classified into one of two families, glycosyl hydrolase family 10 and family 11, formerly F and G, respectively [12]. Xylanolytic microorganisms often synthesize multiple groups of different enzymes, which improves the efficiency of xylan degradation. Like many other polysaccharide-hydrolyzing enzymes, xylanases contain multidomain structures comprising a catalytic domain associated with discrete non-catalytic domains called carbohydrate-binding modules (CBMs). The main functions of CBMs are to facilitate the association of enzymes with substrates enhancing their degradative activity, to mediate protein-protein interactions, and to anchor the enzyme to the cell surface [3]. Like the families of catalytic domains, the CBMs are also grouped into a number of distinct families. Currently, 51 families of CBMs have been identified and characterized [http://www.cazy.org/fam/acc_CBM.html].

Industrial interest in xylan and its hydrolytic enzymatic complex is based on their use as a supplement in animal feed, in the manufacture of bread, food and drinks, textiles, in the bleaching of cellulose pulp, and in ethanol and xylitol production [25]. For commercial applications, xylanases should be produced quickly and in large quantities from simple and inexpensive substrates. Solid-state fermentation (SSF) involves the growth and metabolism of microorganisms on moist solids in the absence or near absence of any free-flowing water [15].

As is the case for yeasts and filamentous fungi, bacteria can grow on solid substrates for SSF and have been used in bioprocesses, both aerobically and anaerobically [29]. SSF can be carried out directly with abundant low-cost biomaterials (including starch, cellulose, lignin, hemicellulose, and chitin) with minimal or no pre-treatment, and is therefore relatively simple, uses less energy, and can provide unique microenvironments conducive to microbial growth and metabolic activities. SSF also has advantages over submerged cultivation and has been used for the production of cellulolytic and xylanolytic enzymes. At the end of the fermentation, the enzymes can be extracted from the substrate easily and inexpensively by percolating the bioreactor with the appropriate buffers [11].

This study describes the cloning, characterization, and overexpression of the modular xylanase Xyl30 from *Streptomyces avermitilis* in *Streptomyces lividans*. The biochemical features and high yields of xylanase produced by SSF confirm successful previous results in which this enzyme was used in the preparation of wheat flour dough and also suggest the commercial potential of this approach in association with lignocellulosic biomass transformation.

Materials and methods

Bacterial strains and plasmids. *Streptomyces avermitilis* CECT 3339, a hemicellulolytic actinomycete isolated in our laboratory, was used as DNA source for the cloning of genes encoding xylanolytic enzymes. *Streptomyces lividans* 66 (John Innes Centre strain number 1326) was used in all cloning experiments carried out in *Streptomyces*. Routine cloning and subcloning were done in *Escherichia coli* DH5a (CECT 4939). DNA fragments were manipulated by cloning into the plasmids pIJ2925 [14] and pN702GEM3 [7].

Media, culture conditions, and sampling procedures. *E. coli* was grown at 37ºC in Luria Broth (LB) containing carbenicillin (100 µg/ml), if required. R2YE and mannitol soya flour medium (MS) agar plates were used for *Streptomyces* growth and sporulation [16]. Transformants of *S. lividans* were selected on BMM-xyl, a modification of the basal mineral medium (BMM) [5], supplemented with 1% insoluble arabinoxylan, 15 µg neomycin/ml, and 1.7% agar. Insoluble arabinoxylan was prepared from oat spelt xylan (Sigma; product number X-0627) according to the method of Sun et al. [30]. For growth studies in liquid medium, *S. lividans* clones were grown in baffled flasks containing 0.1 volume of BMM supplemented with 15 µg neomycin/ml and either 1% arabinoxylan (oat spelt xylan; Sigma, product number X-0627) or glucose (BMM-liq/xyl or BMM-liq/glu, respectively). The media were inoculated with 10^6 colony-forming units (CFU)/ml and incubated in an orbital shaker at 28°C and 250 rpm for as long as required for each assay (1–7 days). One-ml samples were removed from the culture at the specified intervals, centrifuged for 5 min at 10,000 ×*g*, and used for the measurement of extracellular xylanase activity and protein content.

Solid-state fermentation (SSF) experiments were carried out using wheat bran (WB) (*Triticum aestivum* var. *astral*), rye bran (RB) (*Secale cereale*), and water-washed rye bran (WWRB). These substrates were locally obtained, air dried, and pulverized to 40-mesh size. WWRB was prepared by washing pulverized rye bran with water at 90ºC until no starch was visualized, as determined using Lugol's iodine staining reagent. Autoclaved bran cereals (121ºC for 15 min) were inoculated with spore suspensions in BMM medium (10^7 CFU/ml) in a proportion of 7:4 (v/v). Petri dishes (150 mm diameter) containing 10 g of these mixtures were incubated at 28°C for 10 days. Conditions of high humidity were maintained by placing a tray of water on the bottom of the incubator. Culture dishes were removed at the specified intervals for enzyme extraction. The enzyme was extracted by mixing the culture medium from dishes with 100-ml aliquots of 50 mM phosphate buffer (pH 7.5) containing 0.1% Tween-80 and 2 M NaCl, and incubating the suspension on an orbital shaker at 200 rpm for 2 h. The suspended slurry was filtered, centrifuged at 15,000 ×*g* for 20 min, and dialyzed overnight against 50 mM phosphate buffer (pH 7.5) at 4ºC. The clear filtrate thus obtained was used in the enzyme assay. Each batch was prepared in triplicate and average values plus percent standard deviations of the mean were obtained.

DNA manipulation, sequencing, and computer-assisted sequence analysis. *Streptomyces* total genomic and plasmidic DNA was obtained as described by Kieser et al. [16]. *S. lividans* protoplasts were obtained from cells grown for 30 h in YEG (1% yeast extract, 1% glucose, 10.3% sucrose, pH 7) supplemented with 5 mM $MgCl_2$ and 0.5% glycine [7]. The methods described by Sambrook et al. [28] were used for the construction, isolation of recombinant DNA, and Southern blotting. In order to clone xylanases homologous to Xys1 from *Streptomyces halstedii* [26], a probe from the encoding *xysA* gene was prepared. The DNA probe consisted of a 505-bp *Sac*I-*Pst*I gene fragment encoding the highly conserved catalytic region of family F/10 xylanases; it is referred to as pXX_s. Restriction and ligation were carried out with enzymes from GIBCO BRL under the conditions recommended by the supplier.

DNA sequencing was done using a Taq dye deoxy terminator cycle sequencing kit and the ABI 373A sequencing system (Applied Biosystems, San Diego, CA). The sequenced DNA and its deduced protein were characterized using the following software: Frame Plot V2.1 [13], PeptideStructure (GCG Wisconsin Package), GC-Profile [9], SignalP V3.0 [1], ProtParam [10], Pfam [8] and PROSITE [6]. Amino acid and protein sequence homology searches in the databases were done using the BLAST program at the NCBI.

Transcription of the *hd30* gene in *S. lividans* transformants was analyzed by RT-PCR using the internal primers RT-hd30-L 5′-AAGGAGCGGATGTTGATGAT-3′ and RT-hd30-R 5′-TCATCTCCATCGACCAGGA-3′ and a RT-PCR kit (One-step RT-PCR Kit, Qiagen). The functionality of the primers for RT-PCR was demonstrated by PCR using genomic DNA as the template. The amplification reaction was carried out for 40 cycles with an annealing temperature of 57ºC. Total RNA was isolated from 3-day-old transformants grown in BMM-liq/xyl and BMM-liq/glu media using a modified Kirby mix protocol [16]. The absence of contaminating DNA in the RT-PCR was confirmed by a control PCR using RNA as a template.

Enzyme assays and protein determination. Unless otherwise stated, all enzyme assays were done at 40°C in 50 mM sodium phosphate buffer pH 7.5. Xylanase activity was determined using 0.5% (w/v) soluble arabinoxylan as the substrate according to the bicinchoninate/Cu^{2+} method [4]. Soluble arabinoxylan was prepared from oat spelt xylan (Sigma, product number X-0627) as described by Sun et al. [30]. Nonenzyme and xylan controls were routinely run. One unit (U) of enzyme activity is defined as the amount of enzyme producing 1 μmol xylose per min. Protein concentrations were measured using the Bradford method with bovine serum albumin (BSA) as the standard.

SDS-PAGE analysis. SDS PAGE was carried out on a 12.5% polyacrylamide gel according to the Laemmli method. After electrophoresis, the gel was stained with Coomassie brilliant blue R. Xylanase zymograms were obtained using 0.1% soluble arabinoxylan copolymerized with 10% polyacrylamide and 0.1% SDS.

Determination of the amino-terminal polypeptide sequence. The xylanase forms Xyl30 "h" and "l" were separated by SDS-PAGE, transferred overnight onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA), and cut out. The amino termini were sequenced by automated Edman degradation in an Applied Biosystems 473A Protein Sequencer.

Xylan-binding assays and enzyme purification. Xylanase Xyl30 affinity for insoluble polysaccharides was studied using insoluble arabinoxylan and microcrystalline (Avicel; Sigma, product number 11365) or fibrous cellulose (α-cellulose; Sigma, product number C-8002). An aliquot of 0.25 mg of protein obtained from the culture supernatant was mixed with 50 mg of polysaccharide in 50 mM sodium phosphate buffer pH 7.5 and incubated at 4°C for 30 min. The samples were shaken every 10 min and subjected to centrifugation. The amount of enzyme remaining in the supernatant was determined by the standard xylanase assay method. The activity lost from the supernatant was assumed to be the activity bound to the insoluble substrate used in each experiment.

Xylan-binding xylanase was partially purified by incubating 10 mg of total protein from the culture supernatant with 1 g of insoluble arabinoxylan in 20 ml of 50 mM sodium phosphate buffer pH 7.5, as above. The xylan-bound protein complex was washed four times with one volume of the same buffer and the protein eluted with two volumes of the same buffer supplemented with 2 M NaCl. The eluted xylanase was dialyzed against the buffer without NaCl, assayed for xylanase activity, and freeze-dried.

Determination of optimal temperature and pH, thermostability and pH stability. Xylanase activity was measured at pH values ranging from 3 to 9 under standard assay conditions. The buffers used were citrate (0.05 M; pH range of 3.0–5.6), phosphate (0.02 M; pH 6.0–8.0), and 100 mM glycine-NaOH (pH 9). Enzyme activities were also assayed under standard conditions at temperatures ranging from 20 to 80ºC.

The thermostability was determined at 30, 40, 50, 60, 70 and 80ºC after incubation of suitably diluted enzyme samples in the absence of substrate for 0, 15, 30, 45, and 60 min. To study the stability of partially purified xylanase at different pHs, appropriate dilutions of xylanase were made in the above buffers in the range of pH 3–9 for 0, 15, 30, 45, and 60 min. After incubation, samples were analyzed immediately under standard conditions for residual xylanase activity.

Viscometry. Viscosimetric assays were carried out by monitoring the flow time of 0.8% soluble arabinoxylan, in 50 mM phosphate pH 7.5 buffer, incubated for 1 h with 0.05–2 U of Xyl30 xylanase, with commercial xylanase from *Thermomyces lanuginosus* (Sigma, product number X-2753) or β-xylosidase from *Aspergillus niger* (Sigma, product number X-3501) serving as controls. The flow-time of the reaction mixture was determined using an Oswald viscometer at 40°C, together with the content of the reducing sugar [4].

Statistical analysis. All experiments were repeated at least three times. The data shown were subjected to statistical analysis using the SPSS 15.0 program.

Nucleotide sequence accession number. The nucleotide sequence data reported in this work are available from the GenBank NCBI database under accession number AF121865.

Results

Cloning and sequencing of *xyl30* and characterization of the deduced product. Southern blot analysis of *S. avermitilis* chromosomal DNA digested with *Bam*HI using the pXX_8 probe revealed a band of about 7.5 kb. This DNA band was purified from agarose gels, ligated into the *Bam*HI site of plasmid pIJ2925, and transformed into *E. coli.* The insert-carrying clones were selected by Southern blot hybridization using the same probe and the selected plasmid containing the insert was denominated pLV30. The insert was digested with several restriction enzymes and a 3.6-kb *Bam*HI-*Xba*I positive fragment was selected against the hybridization probe and then subcloned into pIJ2925 (plasmid pLV30a). The *Bam*HI-*Xba*I DNA fragment was sequenced (3588 bp) in both directions (Fig. 1).

Analysis of the DNA sequence showed two complete ORFs in opposite directions, ORF1, with 1317 bp and denoted *xyl30*, had an initiation codon (ATG) at nucleotide (nt) 128 and a stop codon (TGA) at nt 1442. On the complementary strand, ORF2, with 1820 bp and denoted *hd30*, had an initiation codon (ATG) at nt 3483 and a stop codon (TGA) at nt 1663 (Fig. 1). The base composition of these genes (69 and 73% G+C) is consistent with the G+C content of *Streptomyces* DNA.

The *xyl30* ORF encodes a deduced peptide (Xyl30) of 438 amino acids (aa) with an estimated molecular mass and pI of 47 kDa and 6.3, respectively. SignalP computer analy-

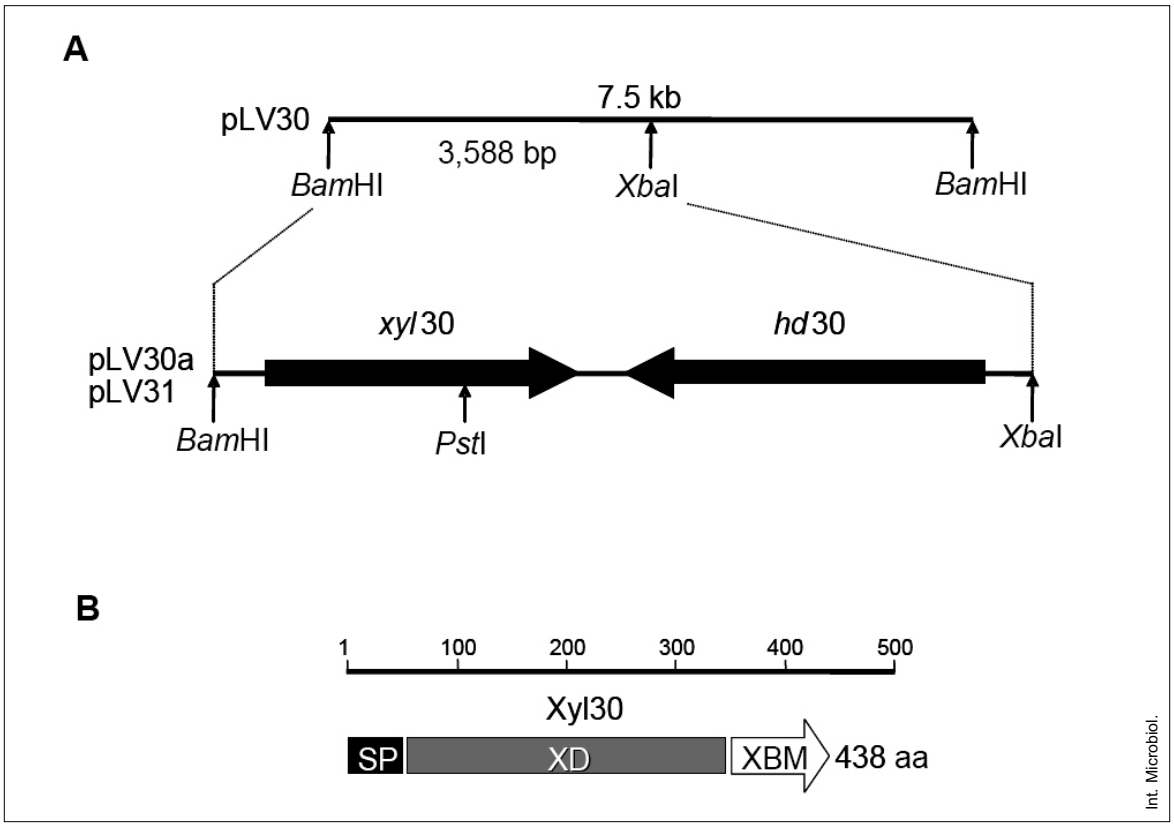

Fig. 1. (**A**) Physical map of the *xyl30-hd30* DNA region cloned from *Streptomyces avermitilis*. pLV30: 7.5-kb *Bam*HI DNA fragment cloned into pIJ2925. pLV30a and pLV31: 3.6-kb *Bam*HI-*Xba*I DNA fragment cloned into pIJ2925 and pN702GEM3, respectively. (**B**) Structure of Xyl30. SP, signal peptide; XD, xylanase domain; XBM, xylan-binding module.

sis showed a putative signal peptide at the N-terminus, with the most likely cleavage site located between positions 42 and 43 and resulting in two deduced peptides (ProtParam) of 4.2 kDa (pI = 12.3) and 42.8 kDa (pI = 5.5). The instability index for these peptides was computed (ProtParam) to be 51.61 and 29.44, thus classifying the proteins as unstable and stable, respectively. PROSITE and Pfam analyses detected the presence of a glycosyl hydrolase family 10 motif (PS00591; aa 271–281) and a glycosyl hydrolase family 10 domain (PF00331; aa 46–342), respectively. A Gly/Pro-rich linker sequence was detected downstream of the xylanase motif at aa 344. Finally, the PROSITE and Pfam analyses also detected a lectin domain of ricin B chain profile (PS50231; aa 367–429) and a ricin B lectin domain (PF00652; aa 358–435), respectively. The deduced amino acid sequence of Xyl30 was compared with that of data bank proteins. Identities of 83, 83, and 79%, with the xylanases A of *Streptomyces coelicolor* A3(2), XlnA of *S. lividans*, and STX-I of *Streptomyces thermoviolaceus*, respectively, were determined. Alignment of the deduced Xyl30 with respect to other family F/10 xylanases (data not shown) identified highly conserved sets of amino acids (aa 165–170 and aa 271–281), and three histidine residues, His^{173}, His^{178}, and His^{249}. An aminoacid sequence homology search in the data banks for the last 94 amino acids containing the ricin B lectin domain revealed identities of 79, 75, and 75% with the carboxylic end of the xylanase SoXyn10A from *Streptomyces olivaceoviridis*, xylanase A from *S. coelicolor*, and xylanase XlnA from *S. lividans*, respectively. All of these xylanases are capable of binding to insoluble xylan.

The *hd30* ORF encodes a deduced peptide (Hd30) of 606 aa, with an estimated molecular mass and pI of 64 kDa and 7.3, respectively. PROSITE and Pfam analyses of the deduced amino acidic sequence of Hd30 detected the presence of a glycosyl hydrolase family 3 motif (PS51318; aa 310–327) and a glycosyl hydrolase family 3 domain (PF00933; aa 124–354), respectively. The Hd30 sequence was compared with that of data bank proteins; an identity of 86% with a putative secreted glycosyl hydrolase of *Streptomyces ambofaciens* and *S. coelicolor* was determined.

Xylanase Xyl30 overproduction in *Streptomyces lividans*. The 3588-bp *Bam*HI-*Xba*I fragment

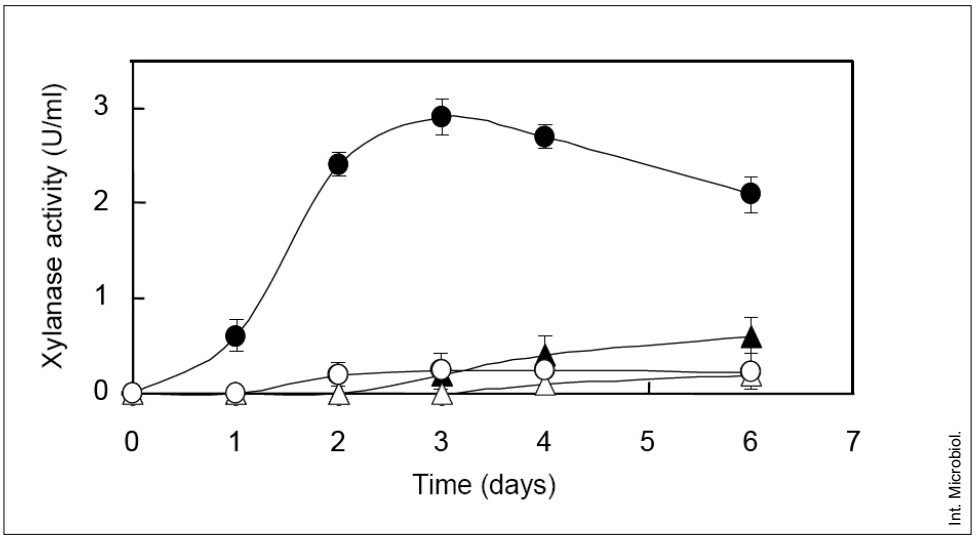

Fig. 2. Xylanase production profile in xylan (xyl)- or glucose (glu)-supplemented BMM liquid medium by *Streptomyces lividans*-Xyl30 (Xyl30) and *S. lividans*-pN702GEM3 (control strain, CS). Symbols: Xyl30-xyl, closed circles; Xyl30-glu, open circles; CS-xyl, closed triangles; CS-glu, open triangles.

containing the putative *xyl30* and *hd30* genes was introduced into the pN702GEM3 plasmid digested with the same enzymes and the resulting plasmid was then used to transform *S. lividans* protoplasts (Fig. 1). Ten xylanase-overproducing clones were selected on solid BMM-xyl after 6 days of incubation at 28°C for the presence of clear areas surrounding the colonies, indicating xylanase activity. All the plasmids of these positive clones carried the same 3.6-kb *Bam*HI-*Xba*I insert. The plasmid was denominated pLV31 and one of the clones (*S. lividans*-Xyl30) was selected for further experiments. The liquid culture of this clone and that of the corresponding control, *S. lividans* carrying the pN702GEM3 plasmid, were carried out in BMM-liq/xyl or BMM-liq/glu.

RT-PCR was done using total RNA samples from *S. lividans*-Xyl30 grown in BMM-liq/xyl or BMM-liq/glu media to assess expression of the *hd30* gene. Under our assay conditions, transcription of *hd30* was not detected (data not shown).

Xylanase activity was detected in the culture supernatants of *S. lividans*-Xyl30 in medium containing arabinoxylan. The highest production of xylanase activity was observed after approximately 3 days of incubation. Very low xylanase production was detected when this strain was grown in medium supplemented with glucose (Fig. 2). Low levels of xylanase production were also detected in the control strain in the presence of arabinoxylan or glucose, probably due to the endogenous production of xylanases in this strain.

SDS-PAGE analyses of the culture supernatants of the producer and the control strains showed that two proteins, of approximately 42.8 and 35 kDa, were expressed in abundance by *S. lividans*-Xyl30. These proteins, named "h" and "l", respectively, were absent in the control strain. Time course SDS-PAGE profiles of xylanase production in *S. lividans*-Xyl30 revealed the presence and ratio of both forms of xylanase. Thus, the heavier "h" protein band (42.8 kDa) was observed after 12 h of culture, while the lighter "l" band (35 kDa) began to appear 12 h later. The concentrations of both forms increased with the fermentation time (Fig. 3A). In addition, zymogram analysis of *S. lividans*-Xyl30 supernatant on renatured SDS PAGE indicated that both bands showed xylanase activity (Fig. 3B). The amino acid sequences of the first 20 positions at the amino terminal of the "h" and "l" protein bands were determined. The N-terminal sequence analysis revealed that the two bands share the same amino acid sequence, which is coincident with the expected deduced xylanase Xyl30 (data not shown).

Binding of xylanase to insoluble polysaccharides. To evaluate the ability of both forms of xylanase ("h" and "l") to bind to insoluble polysaccharides, the culture supernatant was incubated with insoluble arabinoxylan, Avicel, or α-cellulose, and the unbound fraction was assayed for xylanase activity. The supernatant lost part of its activity when arabinoxylan was used, in this case 35% of the xylanase activity was bound to insoluble xylan. With Avicel and α-cellulose, xylanase activity remained in the unbound fraction, suggesting that the enzyme was not bound to these polysaccharides. SDS-PAGE of the proteins retained in the arabinoxylan demonstrated that only the Xyl30 "h" band bound to the substrate (Fig. 3C).

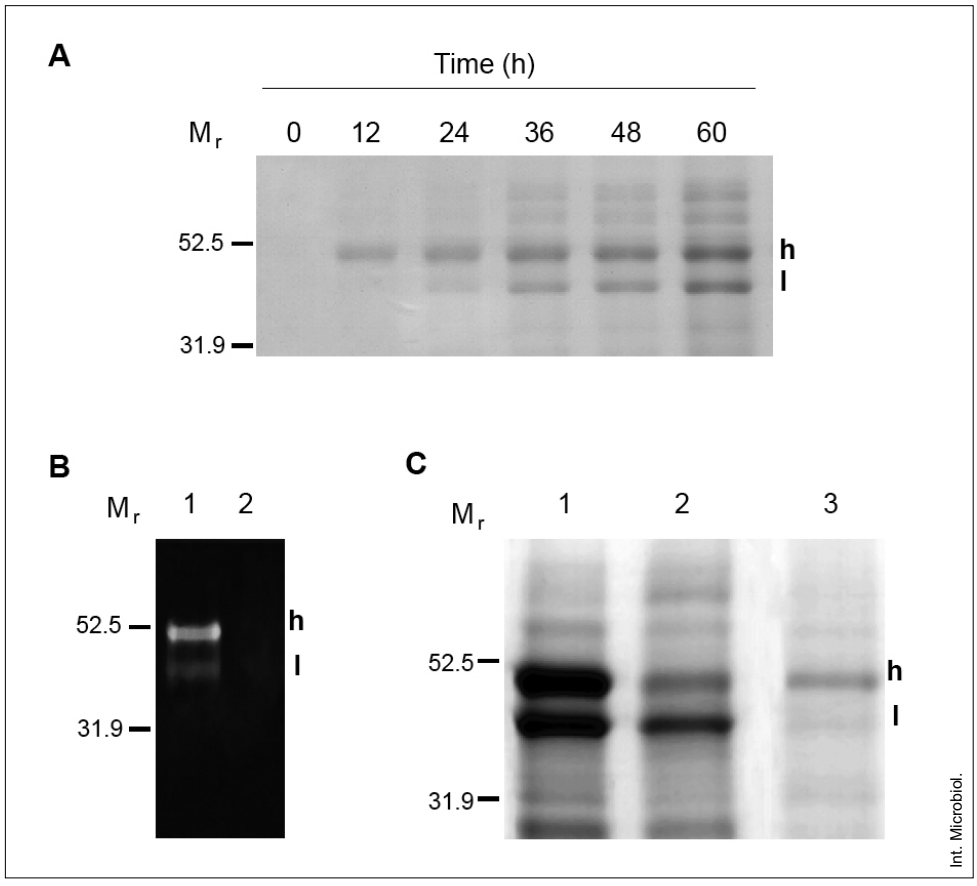

Fig. 3. Electrophoretic analysis of the recombinant xylanase Xyl30. (**A**) Time course SDS-PAGE profiles of xylanase production in *Streptomyces lividans*-Xyl30. (**B**) Zymogram for detecting xylanase activity. Lanes: 1, supernatant of 36-h culture broth of *S. lividans*-Xyl30; 2, supernatant of 36-h culture broth of *S. lividans*-pN702GEM3. h and l: heavier and lighter protein bands, respectively. (**C**) SDS-PAGE of purified xylan-binding xylanase by adsorption-desorption on insoluble xylan. Lanes: 1, culture supernatant; 2, unbound proteins (culture supernatant treated with insoluble arabinoxylan); 3, bound xylanase (eluted from arabinoxylan with 2 M NaCl). Letters "h" and "l": heavier and lighter protein bands, respectively.

Characterization of the xylanase Xyl30. The effect of pH and temperature on the activity of partially purified Xyl30 "h" form was determined. The enzyme was most active at neutral-basic pH, showing highest activity at pH 7.5, with more than 50% of its maximum activity at pH values between 3.0 and 9.0. The optimum temperature for activity was 60°C, while more than 50% of maximum activity was detected between 20 and 80°C.

Stability assays showed that xylanase is highly stable between pH 4.0 and pH 9.0, retaining more than 60% of its initial activity after 1 h of incubation. The xylanase retained 100% of its activity after incubation at 30 or 40ºC for 1 h. A sharp decrease (up to 50%) in activity was detected after 15 min of incubation at 70 or 80ºC. However, the protein still retained 35% of its activity after a 1-h incubation at 87ºC, which indicates that it is a highly stable protein.

The mode of action of the enzyme was determined by measuring the rate of reducing sugar formation and viscosity reduction of arabinoxylan and comparing this activity with the activity of one of the commercial enzymes. The assay for Xyl30 and the commercial xylanase showed a rapid reduction in viscosity and a corresponding rapid increase in reducing sugar levels in the presence of either enzyme. The results for the commercial β-xylosidase showed the release of similar amounts of reducing sugars but no significant reduction in viscosity. The viscosity reduction using 2 U of the different enzymes was 0.38 for Xyl30, 0.43 for the commercial xylanase, and 0.10 for the β-xylosidase (Fig. 4).

Time course of xylanase production in solid-state fermentation. *S. lividans*-Xyl30 and its control *S. lividans*-pN702GEM3 were used in solid-state fermentation

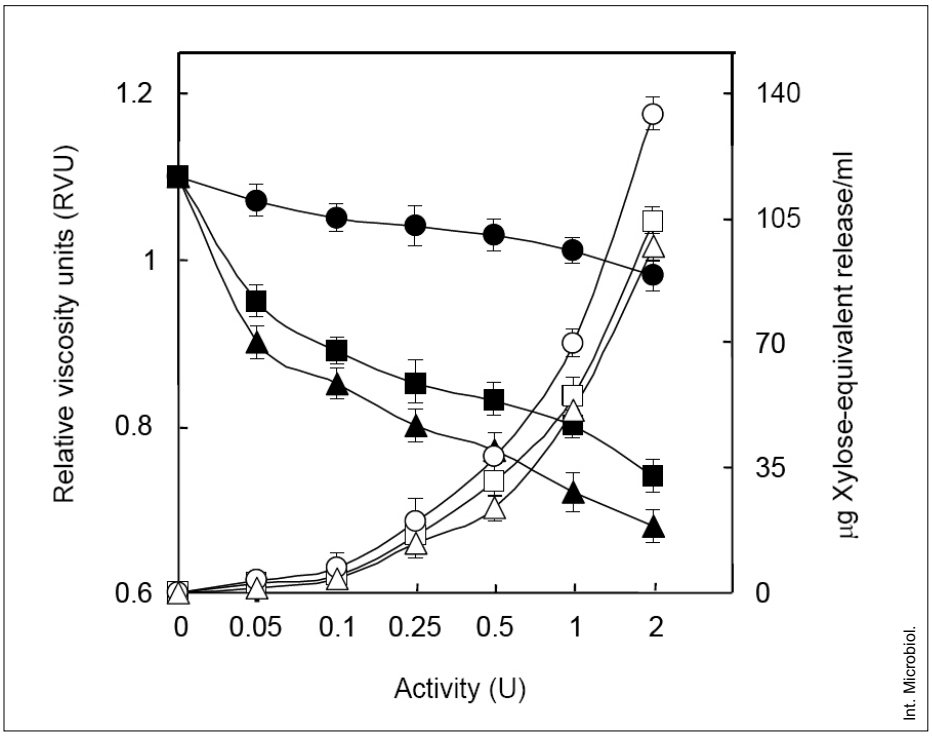

Fig. 4. Diminution in viscosity and formation of reducing-sugar from oat spelt arabinoxylan by three xylanases: xylanase Xyl30 (Xyl30), commercial β-xylosidase (exo-xyl), and xylanase (endo-xyl). Diminution in viscosity (left): Xyl30, closed squares; exo-xyl, closed circles; endo-xyl: closed triangles. Formation of reducing-sugar (right): Xyl30, open squares; exo-xyl, open circles; endo-xyl, open triangles.

(SSF) experiments with wheat bran, rye bran, and water washed rye bran as substrates. As shown in Fig. 5, the xylanase activity of *S. lividans*-Xyl30 was high in the presence of the three fermentation substrates. The maximum production of xylanase occurred after 144 h of incubation using rye bran as substrate. When water-washed rye or wheat bran was used as substrate, maximum production was reached after 216 h. Xylanase activity was also detected when the control strain (*S. lividans*-pN702GEM3) was grown in wheat or rye bran. However, in both cases the activity detected was about one fourth of that detected in the strain carrying Xyl30. Low activity was also detected for the control strain in the presence of water-washed rye bran.

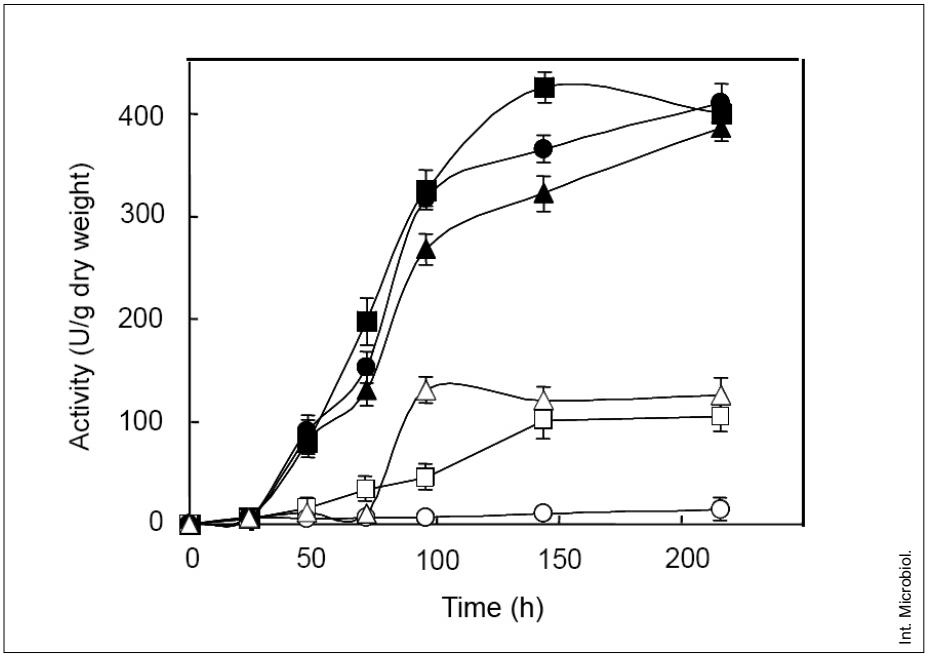

Fig. 5. Time-course profiles of xylanase production in *Streptomyces lividans*-Xyl30 (Xyl30) and the control strain *S. lividans*-pN702GEM3 (CS) in solid-state fermentation (SSF) using wheat bran (WB), rye bran (RB), and water-washed rye bran (WWRB) as substrates. Symbols: RB-Xyl30: close squares; WWRB-Xyl30: closed circles; WB-Xyl30: closed triangles; RB-CS: open squares; WWRB-CS: open circles; WB-CS: open triangles.

Discussion

The present report describes the cloning, sequencing, and *in silico* characterization of a 3.6-kb DNA fragment from *S. avermitilis* CECT 3339. The fragment was isolated using a hybridization probe for xylanases. The cloned DNA fragment encoding the xylanase Xyl30 was expressed in *S. lividans*, partially characterized, and overproduced in SSF. Although a xylanase probe from *S. halstedii* was used, a BLAST comparison based on amino acid sequences showed that Xyl30 had a higher similarity with other xylanases from the same family than with the one used as a probe. This may have been due to the fact that the probe used for Southern blot analysis only contained a highly conserved DNA region of family F/10 xylanases, permitting a selection of encoding sequences of different xylanases from the same family.

In the opposite direction to the *xyl30* gene and located on the complementary strand, a complete ORF encoding a putative secreted hydrolase, designated *hd30*, was detected. RT-PCR analysis showed that *hd30* is not expressed in *S. lividans*-Xyl30 when the cells are grown in BMM-liq/xyl or BMM-liq/glu media. The deduced Xyl30 is a modular protein with two predicted domains, a xylanase family F/10 domain and a xylan-binding domain, and has an estimated molecular mass of 42.8 kDa ("h" form). The carboxy terminus of the deduced Xyl30 is similar to a ricin B lectin domain that is exploited by the xylanases XlnA from *S. lividans* and SoXyn10A from *Streptomyces olivaceoviridis* in order to maintain a reasonably high affinity for β-1,4-linked polymers of xylose [3]. The function of the putative xylan-binding domain of Xyl30 was demonstrated by the high adsorption degree of the "h" form on insoluble arabinoxylan (see Fig. 3C). Thus, this domain appears to be a potential tool for bringing the xylanase directly to the surface of the insoluble hemicellulose containing the lignocellulosic substrate [2].

The Xyl30 xylanase is secreted and some of it processed extracellularly, generating a band of 35 kDa ("l" form) that maintains xylanolytic activity but not the capacity to bind to xylan. Proteolytic processing of actinomycete cellulases and xylanases has been reported by several authors; generally, the substrate-binding domain is removed from the original protein in a linker region to release a functional, catalytic, monodomain enzyme [27]. A putative metalloprotease has been described as being involved in the processing of *Streptomyces reticulii* cellulase [22] and a serine protease implicated in cellulose processing has been cloned and studied in *Thermobifida fusca* [18]. Proteolysis by several serine proteases has also been demonstrated in the xylanase Xys1 from *S. halstedii* when expressed in the original strain or in different hosts, such as *S. lividans*. In vitro experiments with different, extracellular, pure serine proteases from *S. lividans* do not have the capacity to process Xys1 [7], suggesting that new, unidentified proteases should be involved in processing. Although proteolysis is not necessary for the activation of cellulases or xylanases, the existence of a free form of the enzyme may facilitate the hydrolysis of soluble oligosaccharides [27].

The biochemical properties of the partially purified "h" form of Xyl30 indicated that this protein retains more than 50% of its activity under acidic and alkaline conditions (pH 3–9). The optimal pH was determined to be 7.5, while for the closely related XlnA from *S. lividans* and STX-I from *S. thermoviolaceus* it was 6 and 7, respectively [23,31]. The optimal pH for Xys1 from *S. halstedii* is 6.3 [26]. The optimum temperature for Xyl30 is 60ºC. This is similar to the optimal temperature of xylanases XlnA and Xys1 and lower than that of STX-I (70ºC) [30]. However, Xyl30 is more thermostable than Xys1, which is inactivated after a 1-h incubation at 60ºC, while Xyl30 retains 50% of its activity under the same conditions. Furthermore, although no thermostabilizing domain could be shown in Xyl30, the enzyme was particularly active at high temperatures (up to 60ºC). This is also the case for several bacterial, fungal, or phytopathogen hydrolases. These results indicated that Xyl30 is active over a wide range of pH values and temperatures, reflecting the enzyme's ability to degrade plant hemicellulosic materials under a wide variety of environmental conditions.

The endo action of the "h" form of Xyl30 was demonstrated by a reduction in the viscosity of arabinoxylan concomitant with the release of reducing sugars. The assay results were similar to those obtained with a commercial endoxylanase from *Thermomyces lanuginosus*. This was in contrast to a commercial β-xylosidase from *Aspergillus niger*, which showed the lowest ratio between viscosity reduction and an increase in reducing sugars, which is a common feature of this type of enzyme. Our results confirm those of previous studies that focused on the effect of Xyl30 and other different xylanases on the viscoelastic properties of wheat flour dough. In those experiments, we found a decrease in dough viscosity in the presence of Xyl30, probably due to endo-degradation of arabinoxylan chains [unpublished results]. The high-molecular-mass arabinoxylan regulates the characteristics of water-holding capacity, oxidative gelation, and viscosity, and has been known to form more rigid gels than obtained with low-molecular-mass arabinoxylan [19].

The main advantages of SSF include: the use of low-cost residues, low energy demand, simple fermentation equipment, generation of less effluent, extended stability of the

products, high volumetric productivity, higher concentration of the products, and relatively low production costs (for a review, see Krishna [17]). To compare the production of xylanase in liquid medium and SSF, enzyme activity was calculated in units of activity per gram of inducer (arabinoxylan). In this case, maximum xylanase activity produced in liquid medium was 298 U/g arabinoxylan, but in SSF (assuming that arabinoxylan content in rye bran is 12% [32]) it was 3514 U/g arabinoxylan. This means that, in SSF, the activity production is increased 11.8-fold. This high level of xylanase Xyl30 expression in SSF and the biochemical properties of the enzyme open up the possibility for its use in industrial processes, such as biological bleaching of paper pulp, saccharification of xylan in the utilization of lignocellulosic biomass in the production of biofuels, and in baking industries.

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