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Overproduction, Purification, and Biochemical Characterization of a Xylanase (Xys1) from *Streptomyces halstedii* JM8

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***Streptomyces halstedii* JM8, isolated from straw, produces and secretes into the culture supernatant at least two proteins with hydrolytic activity towards xylan. The cloning of a DNA fragment of this microorganism in several *Streptomyces* strains permitted us to overproduce both proteins. N-terminal sequence analyses, immunoblot assays, and time course overproduction experiments allowed us to ensure that both xylanases were encoded by the same gene and that the smallest form (35 kDa) originated from the large one (45 kDa) by proteolytic cleavage on the C terminus. The production of both forms was studied in different strains carrying the gene in a multicopy plasmid. The best production was obtained with *Streptomyces parvulus* transformed with the plasmid pJM9, a pIJ702 derivative, which yielded 144 U/ml. Both forms of the xylanase were purified with a fast-performance liquid chromatography system and characterized biochemically. The optimal pH and temperature, for both, were 6.3 and 60°C, respectively, in 7.5-min assays. Both proteins were highly stable in a wide range of pHs (4 to 10) and temperatures (4 to 50°C); nevertheless, after 1-h incubations, both enzymes lost most of their activity at temperatures over 55 to 60°C. Endoxylanolytic activity was demonstrated in both enzymes, but no β-xylosidase activity was detected.**

Xylanases (1,4-β-D-xylanhydrolase; EC 3.2.1.8) catalyze the hydrolysis of xylan to xylo-oligosaccharides and xylose, and these products can be useful feedstock for food and fine chemicals (8). Xylan is the major component of monocotyledon hemicelluloses of plant cell walls (22). β-(1,4)-Linked D-xylose residues constitute a backbone which can also contain arabinose, glucuronic acid, and/or mannose substitutes. Xylan-degrading enzymes are produced by a wide variety of microorganisms, including aerobic and anaerobic mesophiles and thermophiles (reviewed in references 2 and 26). The study of several xylanolytic systems has permitted the corroboration of the fact that, usually, more than one xylanase is produced by each microorganism (5). These multienzymatic systems suggest that every xylanase may have specialized functions to perform a more effective xylan hydrolysis. Despite their industrial applications and their role in the bioconversion of renewable plant cell materials, little is known about the real mechanism of xylanase catalysis, and it has been suggested that xylanases act by general acid catalysis involving amino acid carboxy groups (3, 19).

The use of xylanases in conjunction with cellulases for the complete conversion of cellulosic biomass to sugars has been widely studied (2), and it can greatly reduce costs in processing lignocellulose biomass (8); even the high specificity of enzyme reactions and the absence of substrate loss due to chemical modifications make advantageous the use of microbial enzymes in the industrial hydrolysis of lignocellulose. On the other hand, pulp industries are considering the use of xylanases to degrade xylan and thereby remove lignin, to bleach paper pulp. This strategy also avoids the use of chemical processes which are very expensive and polluting (11). In this paper, we report the overproduction, purification, and biochemical characterization of two forms of a new xylanase isolated from *Streptomyces halstedii* JM8.

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MATERIALS AND METHODS

Strains and plasmids. *S. halstedii* JM8 was isolated from straw in a screening for microorganisms with cellulolytic and/or xylanolytic activities (7). *Streptomyces albus* G″R 2″ J1074, *Streptomyces lividans* JI 66, and *Streptomyces parvulus* JI 2283 (from The John Innes Institute, Norwich, England) were used as host organisms.

All of the plasmids used in this work were derived from vectors pIJ702 (12) and pIJ487 (25). pJM9 and pJM10 were obtained from the initial library, generated by partial digestion with *Sau*3A of *S. halstedii* JM8 DNA, and subsequently cloned in pIJ702 (7). pXA3 arises from subcloning in pIJ487 of a 3-kb DNA *Bam*HI-*Bgl*II fragment from pJM9 insertion (Fig. 1).

Culture medium and growth conditions. To standardize the liquid cultures, plates of R2YE medium (supplemented with 20 μg of thiostrepton per ml when needed) (10) were inoculated with different *Streptomyces* species and incubated at 28°C. The spores were harvested after 2 weeks as described in *Streptomyces* protocols (10). Spore counting was carried out with a cell-counting chamber (Thoma chamber; Brand, Wertheim, Germany), and the spores were stored in 20% glycerol at −20°C. All liquid cultures were inoculated to yield 1.4×10^6 spores per ml in 250-ml four-baffled flasks containing 25 ml of YES medium (1% yeast extract, 10.3% sucrose [pH 7.2]) supplemented with 0.5% oat spelt xylan (Sigma) and 5 mM $MgCl_2$; thiostrepton (5 μg/ml) was added when needed. Incubation was carried out at 28°C and with shaking at 250 rpm in an orbital shaker (Adolf Kühner AG, Birsfelden, Switzerland). Samples (1 ml) were harvested every 24 h, and the supernatant was kept frozen until used.

Preparation and purification of xylanase. To purify both forms of xylanase, supernatant from 600 ml of 84-h cultures of *S. parvulus* carrying pJM9 was separated from the mycelium by centrifugation at 6,000 × *g* for 15 min. The supernatant was filtered through two Whatman no. 3 paper filters and centrifuged at 12,000 × *g* for 15 min. The resulting solution was concentrated in a Minitan system (Millipore) with polysulfone membranes (normal molecular weight cutoff point, 10,000; Millipore). Dialysis of concentrates was performed against distilled water in the same system. The sample (55 ml) was filtered through 0.22-μm-pore-size syringe filters (Gelman Sciences) and brought to pH 6.0 with 10 mM morpholineethanesulfonic acid (MES)-NaOH. The same buffer was used for purification. Purification was performed with a fast-performance liquid chromatography (FPLC) system (Pharmacia) equipped with an anionic-exchange column (Q-HR 5/5; Pharmacia). The column was equilibrated with 10 mM MES-NaOH (pH 6.0), and an NaCl gradient from 0 to 0.15 M was used for protein elution (flow rate, 1 ml/min). The residual protein was eluted with 1 M NaCl. Xylanolytic activity of the different fractions was detected with 1.5% agar plates containing 0.1% Remazol brilliant blue xylan (Sigma).

Protein electrophoresis. Protein concentration was measured by the method of

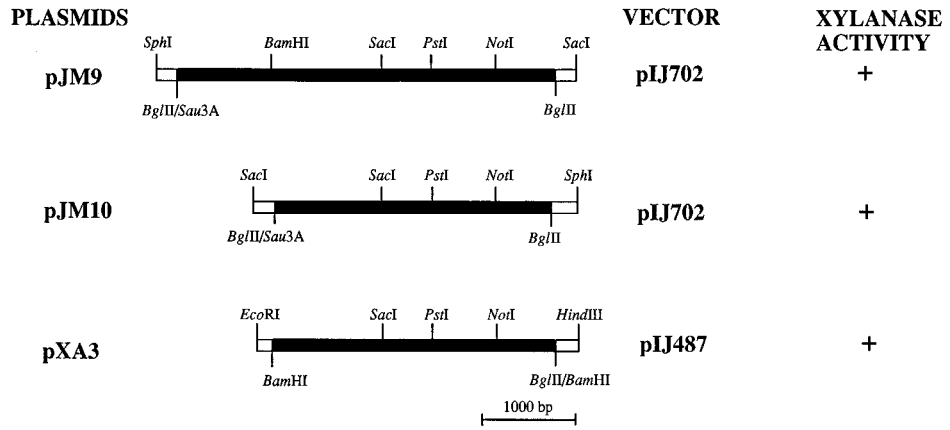

FIG. 1. Partial restriction map of plasmids obtained during this work. Plasmid names are indicated on the left; the vectors used and xylanase activity (+) are indicated on the right. Only the restriction enzymes used to determine the orientation are shown. The scale is adequate only for the insert (▬), not for the vector (▭).

Peterson (20), with bovine serum albumin as the standard. Electrophoresis was always carried out on 15% denaturating polyacrylamide gels (sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) (14). Five or 7 μl of supernatant was equilibrated with sample buffer (14), boiled for 5 min, and loaded in a Mini Protean II system (Bio-Rad). When the protein level was low, 10% trichloroacetic acid precipitation was used. Low-molecular-weight standards from Bio-Rad were used as size markers. Coomassie blue R was normally used for protein staining, but sometimes silver staining was carried out (17).

Enzyme assays. All of the assays for biochemical characterization were made with both forms of the purified enzyme resuspended in 10 mM MES-NaOH (pH 6.0). The reducing sugar concentration was measured by the 3,5-dinitrosalicylic acid method (1), with xylose as the standard. The reaction mixture was composed of 100 μl of suitably diluted and previously purified protein, 100 μl of 4% soluble oat spelt xylan (see below), and 200 μl of a suitable buffer (final concentration, 50 mM) for each assay. Substrate and enzyme controls were always used. Conditions for the reaction were 60°C and 7.5 min. The reaction was stopped with 1 ml of 3,5-dinitrosalicylic acid, and the reaction mixture was boiled for 5 min and diluted with 4 ml of water. The samples were centrifuged at 5,000 × *g* for 5 min, and the A_{540} of supernatant was measured. One unit of enzymatic activity was defined as the amount of enzyme which releases 1 μmol of product (xylose) in 1 min of reaction under the established conditions.

Effects of pH and temperature on xylanase activities and stabilities. Estimations of xylanase activities at different pHs and temperatures were done for both purified forms. To determine the optimal pH, the range from 3 to 10.5 was used with the following (50 mM) buffers: sodium citrate (pH 3 to 6), sodium phosphate and Tris-maleate-NaOH (pH 6 to 8), Tris-HCl (pH 8 to 9), and glycine-NaOH (pH 9 to 10.5). For pH stability, the enzyme was preincubated for 1 h at 32°C in a suitable buffer, and later, the substrate was added and the reaction occurred in 50 mM citrate-phosphate buffer (pH 6.3) for 7.5 min at 60°C.

Optimal temperature and thermostability were determined in the range from 4 to 80°C in 50 mM citrate-phosphate buffer (pH 6.3). To determine the thermal stability, the proteins were preincubated for 1 h at the corresponding temperature, and later, the substrate was added and the reaction occurred for 7.5 min at 60°C.

Analysis of xylan degradation products. Xylan degradation was carried out with 1 U of xylanase and 10 mg of soluble xylan in 1 ml of 50 mM citrate-phosphate buffer (pH 6.3). Samples of 50 μl were withdrawn periodically, and 3 μl of a 1/5 dilution was spotted onto thin-layer chromatography Kiesegel 60 layers (Merck, Darmstadt, Germany) and chromatographed at room temperature in a solvent system containing acetonitrile-water (72:28 [vol/vol]) (6). The products of hydrolysis released were located by the orcinol-sulfuric acid reaction (4).

Substrate specificity. To determine the specificity of the activities of the purified proteins, both forms were assayed on the following substrates: soluble and insoluble fractions of oat spelt xylan (see below), larchwood xylan (Janssen Chimica, Beerse, Belgium), lichenan (Sigma), and low-viscosity carboxymethyl cellulose (Sigma). Reducing sugars were measured by the 3,5-dinitrosalicylic acid method with xylose or glucose as the standard, depending on the sugar present in the polymer. The activities were compared with the highest one obtained for any of these substrates.

β-Xylosidase activity was tested with *p*-nitrophenyl-β-D-xylopyranoside (Sigma) as the substrate. The reaction mixture consisted of 1 ml of 50 mM citrate-phosphate buffer (pH 6.3), 200 μl of 25 mM *p*-nitrophenyl-β-D-xylopyranoside (Sigma), and 20 μl of the appropriate protein dilution for 1 h at 60°C. The reaction was stopped by adding 2 ml of 2 M Na_2CO_3 (Merck). Suitable substrate and enzyme blanks were established. Activity was determined as color generation by measuring the A_{405}. One unit of enzymatic activity was defined as the amount of enzyme which releases 1 μmol of *p*-nitrophenyl in 1 min of reaction under the established conditions.

Soluble xylan. The soluble fraction of commercial xylan (oat spelt xylan; Sigma) was used as the substrate for all assays. The commercial xylan was shaken in distilled water for 24 h at room temperature. Insoluble and soluble fractions were separated by centrifugation. They were then lyophilized to complete dryness.

Amino-terminal amino acid sequences. Both xylanase forms were separated by SDS-PAGE, blotted onto Immobilon P (Millipore) membranes, and cut off. The amino terminus was sequenced with an Applied Biosystems 470A Protein Sequenator.

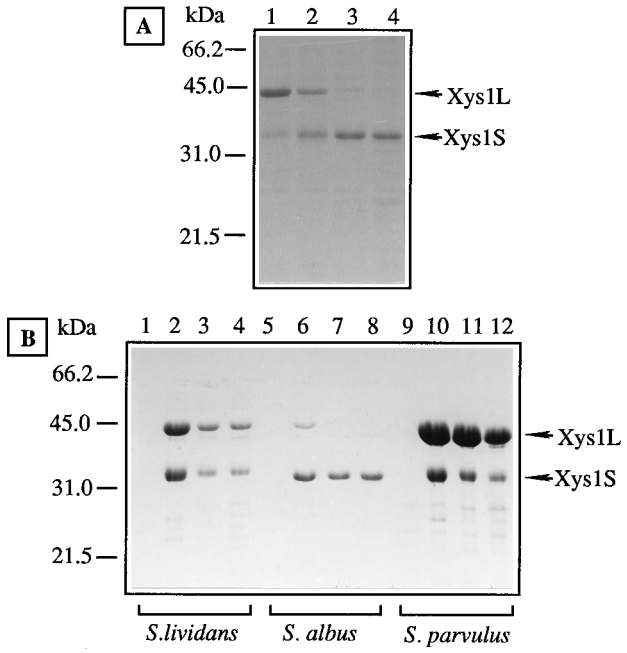

FIG. 2. Coomassie blue-stained SDS-polyacrylamide gel of 7 μl of culture supernatant. (A) Processing of the protein Xys1L in *S. lividans*/pJM10 liquid culture time course. Lanes: 1, 48 h; 2, 84 h; 3, 120 h; 4, 156 h. (B) Comparative production of the proteins Xys1L and Xys1S by several *Streptomyces* hosts carrying the plasmids pIJ702 (lanes 1, 5, and 9), pJM9 (lanes 2, 6, and 10), pJM10 (lanes 3, 7, and 11), and pXA3 (lanes 4, 8, and 12).

TABLE 1. Production of xylanase (Xys1) by several *Streptomyces* hosts

Strain	Supernatant protein concn (mg/ml)	Xylanase activity	
		U/ml	U/mg
S. halstedii JM8	0.1286	4.12	32.03
S. lividans/pIJ702	0.1475	1.00	6.78
S. lividans/pJM9	0.8846	113.25	128.02
S. lividans/pJM10	0.6335	62.25	98.26
S. lividans/pXA3	0.7803	60.50	77.53
S. albus/pIJ702	0.1417	2.00	14.11
S. albus/pJM9	0.6247	73.00	116.85
S. albus/pJM10	0.4975	53.00	106.53
S. albus/pXA3	0.4387	57.75	131.63
S. parvulus/pIJ702	0.2787	0.25	0.89
S. parvulus/pJM9	1.5917	144.00	90.46
S. parvulus/pJM10	1.3226	126.00	95.26
S. parvulus/pXA3	1.2790	104.75	81.31

RESULTS

Selection of *S. lividans* clones overproducing xylanase. After the screening of a genomic library of *S. halstedii* JM8 in *S. lividans* (7), on plates of R2YE containing 0.3% oat spelt xylan, two clones with xylanolytic activity were isolated. The plasmids isolated were named pJM9 and pJM10. In both cases, SDS-PAGE of their supernatants revealed two secreted protein bands of 45 and 35 kDa. N-terminal sequencing showed a complete identity between both proteins (AGALGDAAAA KGR). Moreover, antibodies raised against the large band strongly recognized the 35-kDa form (data not shown), and in a time course overproduction experiment, processing from the large protein to the small one was observed (Fig. 2A). The 45-kDa enzyme is produced first, and in aged cultures it is proteolytically cleaved at its carboxy terminus, producing the smaller form, which is also active. The large protein was named Xys1L, and the small one was named Xys1S.

Xylanase production and vector and host effects. Several laboratory *Streptomyces* strains were used to find adequate production of both peptides. The plasmids pIJ702 (vector), pJM9, pJM10, and pXA3 (a pIJ487 derivative) (Fig. 1) were transformed in *S. lividans*, *S. albus*, and *S. parvulus*, and all of the cultures were carried out under the conditions described in Materials and Methods. The cultures were grown for 86 h at 28°C and at 250 rpm. Seven microliters of the resulting supernatants were loaded on an SDS–15% polyacrylamide gel to analyze xylanase production. Under the established conditions (YES medium supplemented with 0.5% xylan), *S. parvulus* transformed with pJM9 plasmid was the best producer (Fig. 2B and Table 1). This strain produces (in absolute values) up to 1.28 times more xylanase than *S. lividans* does and twice that produced by *S. albus* transformed with the same plasmid. Proteolytic processing of Xys1L to yield Xys1S was observed in all cases, but this processing was more effective in *S. albus* transformants in which most of the produced protein is in the processed form. This proteolytic cleavage occurred even in early stages in a time course experiment (data not shown).

Purification of both enzymes. *S. parvulus* carrying pJM9 was grown for 84 h in 600 ml of YES medium supplemented with 0.5% xylan, and the supernatant was concentrated 12-fold and

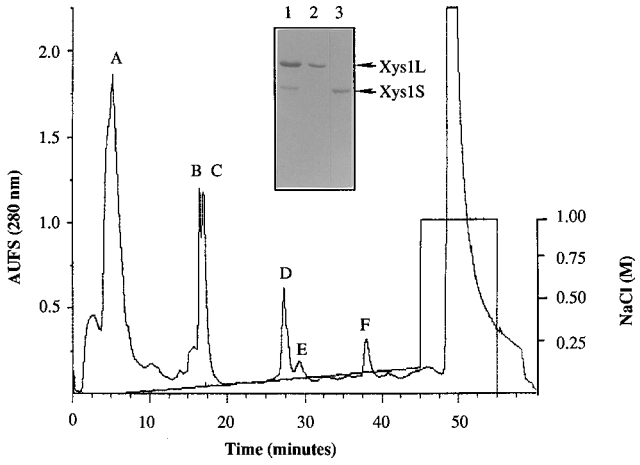

FIG. 3. Purification of both xylanases with an anionic-exchange column (Q-HR 5/5). The column was used as described in Materials and Methods. The insert shows a Coomassie blue-stained SDS-polyacrylamide gel of the original sample (lane 1), peak A (lane 2), and peak F (lane 3). AUFS, absorbance units full scale.

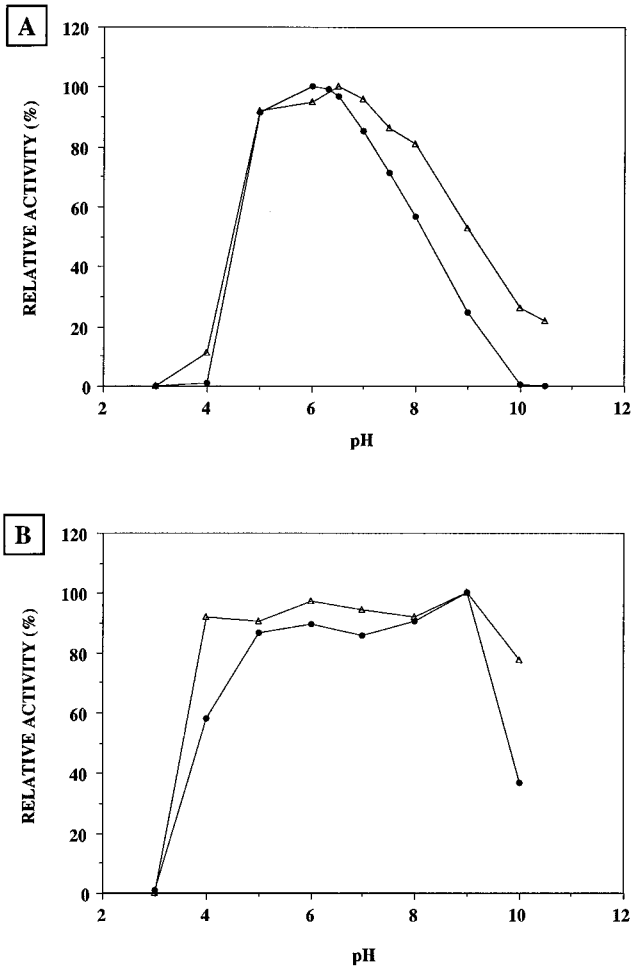

FIG. 4. (A) Effect of the pH on activities of Xys1L (△) and Xys1S (●). The buffers used were sodium citrate from pH 3 to 6, sodium phosphate and Tris-maleate-NaOH from pH 6 to 8, Tris-HCl from pH 8 to 9, and glycine-NaOH from pH 9 to 10.5. The concentration of each buffer in the reaction was 50 mM. (B) pH stability in the same range of pH for Xys1L (△) and Xys1S (●). The enzymes were preincubated for 1 h at 32°C in a suitable buffer for each pH, and later, the reaction was carried out in 50 mM citrate-phosphate buffer (pH 6.3) for 7.5 min at 60°C. The results shown are the results of three different experiments.

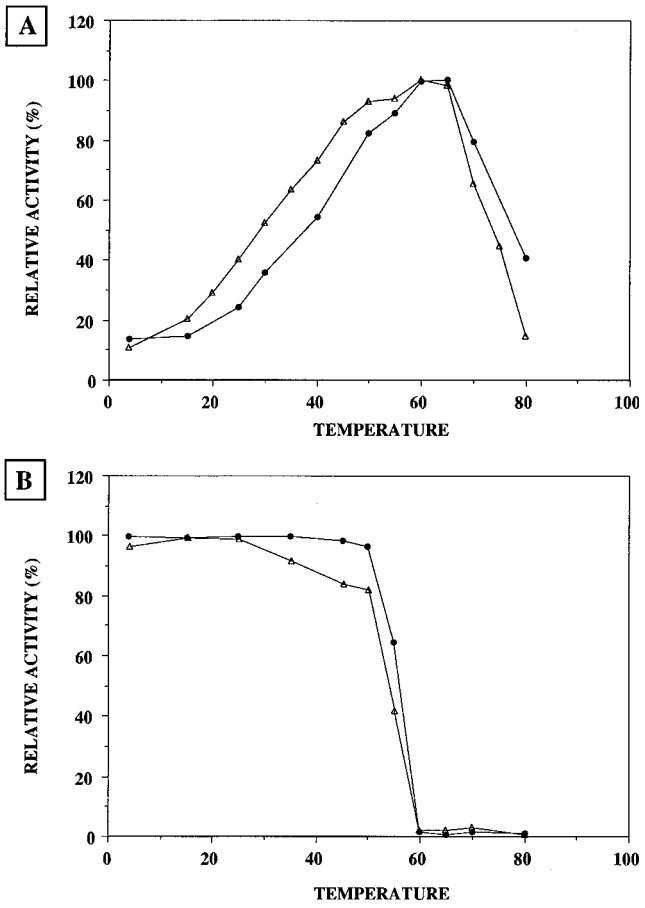

FIG. 5. Effect of temperature on activity (A) and stability (B) of Xys1L (△) and Xys1S (●). For thermostability, the enzymes were preincubated for 1 h at the indicated temperature, and later, the reaction was carried out in 50 mM citrate-phosphate buffer (pH 6.3) for 7.5 min at 60°C. The results shown are the results of three different experiments.

dialyzed in a Minitan system (Millipore). The resulting preparation was filtered through 0.22-μm-pore-size filters and equilibrated with 10 mM MES-NaOH (pH 6.0). Separation of proteins was carried out by anionic exchange in an FPLC system (Pharmacia) under the conditions described in Materials and Methods. Xylanase activity appeared mainly in peaks A and F (Fig. 3), corresponding to the large enzyme (Xys1L) and the processed one (Xys1S), respectively. A Coomassie blue-stained SDS-polyacrylamide gel of the purified enzymes showed a single band in each fraction (Fig. 3, insert).

Biochemical characterization. Both FPLC fractions (Fig. 3, peaks A and F) were lyophilized and dialyzed against 10 mM MES-NaOH (pH 6.0) for 24 h. The protein concentration was determined, and amounts of protein between 1 and 2.5 μg were used in the enzymatic assays. The effects of pH and temperature on the activity and stability of both forms were studied as described in Materials and Methods. Both enzymes have optimal activity at pH 6.3 and at 60°C (Fig. 4A and 5A). For pH stability, a pH range of 3 to 10.5 was used. At least 80% of activity was maintained from pH 4 to 10 after incubation at 32°C for 1 h; however, both enzymes became completely inactive when they were incubated at pH 3 (Fig. 4B). Even very short incubations (2.5 min) at this pH resulted in nonreversible inactivation (data not shown). The stability of the enzymes to temperatures was studied from 4 to 80°C. The enzymes conserve most of their activity from 4 to 50°C. However, incubations for 1 h at higher temperatures inactivated both enzymes (Fig. 5B).

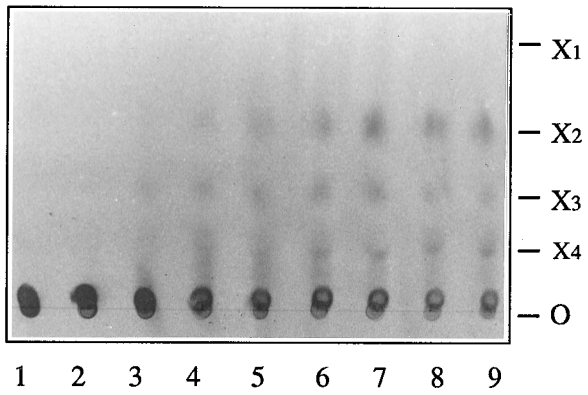

FIG. 6. Thin-layer chromatography of products generated by Xys1L on soluble oat spelt xylan. The conditions are described in Materials and Methods. Samples were analyzed at different times. Lanes: 1, xylan control; 2, digestion for 5 min; 3 to 9, samples taken every 30 min (lanes 3 to 6) or after 4 (lane 7), 5 (lane 8), or 24 (lane 9) h. Abbreviations: X_1, xylose; X_2, xylobiose; X_3, xylotriose; X_4, xylotetraose; O, origin.

Products of xylan hydrolysis. The hydrolysis patterns of soluble xylan by both enzymes were studied by thin-layer chromatography. Both proteins showed an identical endo mode of action. Thus, as xylan degradation occurred, a large amount of intermediate products (xylotetrose, xylotriose, and xylobiose) was obtained, but a very small quantity of xylose appeared. Even for long (24-h) incubation periods, xylobiose was the most abundant product (Fig. 6).

Substrate specificity. The activities of both purified forms were tested on different substrates (see Materials and Methods). Liberation of reducing groups was detected only when xylose polysaccharides were used. The highest activity was detected on the soluble fraction of oat spelt xylan, and it was three times higher than the activity obtained on the insoluble fraction and twice that yielded on larchwood xylan (Table 2). No hydrolytic activity was detected on the glucose polymers.

DISCUSSION

Streptomyces spp., usually present in soil and decaying plant material, have been screened for xylanases (27). *S. halstedii* JM8 was originally selected in our laboratory for its endoglucanase activity (7), but later experiments showed that this microorganism produces xylanases and several other different enzymes. In this paper, we deal with the study of two forms of a xylanase which are produced after the cloning of a fragment of *S. halstedii* DNA in *S. lividans*, *S. albus*, and *S. parvulus*. These two proteins of 45 and 35 kDa, Xys1L and Xys1S, respectively, are secreted into culture supernatants. Since the

TABLE 2. Activity of Xys1L on different substrates

Substrate	% Activity
Soluble oat spelt xylan	100.00
Insoluble oat spelt xylan	33.28
Larchwood xylan	50.34
Lichenan	0.00
CMC (low viscosity)[a]	0.00

[a] CMC, carboxymethyl cellulose.

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Xys1L AGAL-GDAAAAKGRYFGAA
Xys1S AGAL-GDAAAAKGR
XlnA  AESTLGAAAAQSGRYFGTA
STXI  AESTLGAAAAQSGRYFGTA
Xyl1  AESTLGAAAAQSGRYFGTA
Xyl4  AESTLGAAAQQSGYYFGTA
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FIG. 7. Comparison of the amino-terminal sequences of Xys1L and Xys1S from *S. halstedii* with other xylanases from different *Streptomyces* species, namely, XlnA from *S. lividans* (21), STXI from *S. thermoviolaceus* (23), and Xyl1 and Xyl4 from *S. roseiscleroticus* (9). *, amino acid identity.

size of the original pJM10 DNA insert was about 3 kb, it was possible to fit two open reading frames encoding for the two secreted proteins. Nevertheless, in aged cultures (after 48 h in *S. lividans* or *S. parvulus* and 24 h in *S. albus*), the amount of the 35-kDa protein increased while the 45-kDa protein amount decreased. The N-terminal sequence analysis revealed that both enzymes shared the same amino acid sequence. Moreover, in Western blot (immunoblot) assays with the antibodies raised against the large form, the small protein was recognized as strongly as the 45-kDa enzyme. The use of anti-Xys1L antibodies in a supernatant of the original strain, *S. halstedii* JM8, assured us that the processing is not due to an overexpression effect in the hosts used. All of these facts suggest that the cloned fragment of *S. halstedii* JM8 DNA encodes an extracellular xylanase of 45 kDa which is proteolytically cleaved on its carboxy-terminal end to give a 35-kDa active form. In other words, both peptides originate from the same gene. Similar results have been reported previously for other *Streptomyces* hydrolytic enzymes such as α-amylases and xylanases. Morosoli et al. (15) described one of these enzymes in *S. lividans*, namely, a 43-kDa xylanase, XlnA, that is processed on its C terminus, to give a 27-kDa active protein. The same authors described another xylanase, XlnB, with similar processing, but its size, N-terminal amino acid sequence, and pI are rather different from those of Xys1 (13, 16). Comparison of the N-terminal sequence of Xys1 (Xys1L and Xys1S) with those of other published *Streptomyces* xylanases indicated similarity with the following: XlnA from *S. lividans* (21), STXI from *S. thermoviolaceus* (23), and Xyl1 and Xyl4 from *S. roseiscleroticus* (9). Curiously, XlnA, XTXI, and Xyl1 have the same N-terminal amino acid sequences (Fig. 7).

The properties of the proteins are similar to those of other *Streptomyces* xylanases, and the optimal pH and temperature values agree with those previously reported for xylanases XlnA and XlnB from *S. lividans* 1326 (13, 15), xylanase II from *S. cyaneus* (24), STXI from *S. thermoviolaceus* (23), xylanase Xyl4 from *S. roseiscleroticus* (9), and one xylanase from *Streptomyces* sp. strain KT-23 (18). On the other hand, the biochemical characteristics of both forms of Xys1 are similar to each other, i.e., they have optimal activities at a pH around 6 and at 60°C. However, after incubation at pH 10, Xys1L lost only 20% of its activity whereas Xys1S lost 60%.

From the xylan hydrolysis products and substrate specificity, xylanase Xys1 is a typical endoxylanase unable to hydrolyze other glycosidic linkages. The main final product of xylan hydrolysis was xylobiose, although a mixture of xylo-oligosaccharides was obtained when brief hydrolysis occurred.

The high level of expression of these proteins and their biochemical properties open the possibility of their use in some industrial processes such as biological bleaching of paper pulp. With this in mind, the cloning of the gene that encodes Xys1 (Xys1L and Xys1S) in a cellulase-deficient host will be necessary.

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