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Characterization of a major xylanase purified from *Lentinula edodes* cultures grown on a commercial solid lignocellulosic substrate

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Summary. The white-rot basidiomycete *Lentinula* (syn. *Lentinus*) *edodes* (Berk.) Pegler is the dominant edible mushroom cultivated on wood. The major xylanase detected in cultures grown on a commercial oak wood medium was extracted, purified, and characterized. The enzyme was a non-debranching endo-β-D-xylanase (1,4-β-D-xylan xylanohydrolase; E.C.3.2.1.8) highly specific for xylans, with a molecular weight of 41000 (on sodium dodecyl sulfate gels) and an isoelectric point of 3.6. With aspen glucuronoxylan as substrate, the enzyme showed optimal activity at pH 4.5–5.0 and 60° C, with a K_m of 0.66 mg/ml and specific activity of 310 units/mg protein at 40° C. It was capable of hydrolyzing (forming reducing sugars from) 40%–50% of the hydrolyzable linkages in either glucuronoxylan or arabinoxylan. The enzyme produced xylose and major identifiable products in the xylobiose or xylotriose (and presumably larger) size range including xylobiose and xylooligosaccharides, but neither glucuronic acid nor arabinose. Products were also produced from arabinoxylan that appeared to be arabinoxylobiose and arabinoxylotriose.

Introduction

In nature, microbial xylanases and associated enzymes degrade the xylans present in lignocellulose (Wong et al. 1988). These xylans consist of acetylated β-(1,4)-D-xylan, typically bearing arabinosyl- and/or 4-*o*-methylglucuronosyl side chains (Whistler and Richards 1970; Jeffries 1987). The characterization of xylanolytic enzymes is important because of their involvement in industrial bioconversions (e.g. edible mushroom production: Hong 1976a, b; Leatham 1985; Hong et al. 1986) and potential use in new large-scale commercial applications. Some examples of potential applications include biopulping wood (Eriksson 1985; Eriksson and Kirk 1985; Myers et al. 1988), retting flax fibers (Sharma 1987), upgrading or refining (e.g., bleaching: Barnoud et al. 1986; Noé et al. 1986; Kantelinen et al. 1988; Jurasek and Paice 1988) chemical or mechanical pulps (Kirk et al. 1983; Eriksson 1985; Eriksson and Kirk 1985), manufacturing dissolving pulp (Barnoud et al. 1986), increasing animal feed digestibility (Wong et al. 1988), converting lignocellulosic sugars into liquid feedstocks or fuels (Kirk et al. 1983; Eriksson 1985; Jeffries 1985), and processing food (e.g. clarification of juices: Biely 1985; Dekker 1985).

To ensure optimal enzyme production and facilitate purification, the xylanases characterized so far have generally been from liquid-medium-grown cultures using purified xylan as carbon source or induced with methyl-β-L-xylose. However, even though different (iso)enzymes may be induced, or different post-transcriptional modifications may occur, little information is available on the xylanases produced by microorganisms grown on solid lignocellulosic substrates.

The conversion of under-utilized hardwoods into the shiitake mushroom *(Lentinula edodes)* is currently the largest commercial bioconversion process utilizing wood (Ito 1978; Royse and Schisler 1980; San Antonio 1981; Leatham 1982). When grown on commercial wood-containing substrates, this lignin-degrading white-rot basidiomycete (Leatham 1985, 1986) has been shown to produce a complex mixture of extracellular degradative enzyme activities, which include xylanase (Tokimoto et al. 1977; Leatham 1985; Hong et al. 1986). Our initial studies suggest that several enzymes are produced in quantities sufficient for characterization. Here, we describe the isolation and partial characterization of the major β-(1,4)-D-xylanase produced by

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L. edodes cultures grown on a commercial oak wood medium. To our knowledge, this is the first report of a xylanase characterized from a solid-substrate conversion process utilizing wood.

Materials and methods

Chemicals. Enzyme substrates and other chemicals were obtained from the following sources: deacetylated glucuronoxylan prepared by KOH extraction of aspen (*Populus tremuloides* Michx.) wood (Dutton and Murata 1961) was provided by R. W. Scott of Forest Products Laboratory; xylooligosaccharides were provided by Robert Hespell of the Northern Regional Research Laboratory, Peoria, Ill, USA; acid-swollen cellulose as prepared from Solkafloc SW-40 (a wood pulp cellulose) from Brown* (Berlin, NH, USA) by the method of Walseth (1952); laminarin from Cal Biochem Behring (La Jolla, Calif, USA); *p*-nitrophenol-β-D-xylopyranoside from Koch-Light Laboratories (Haverhill, Suffolk, UK); arabinoxylan (deacetylated oat spelt xylan), polyethylenimine (50000 molecular weight, 50% aqueous solution), high-viscosity carboxymethylcellulose, protein molecular weight standards, and all other chemicals from Sigma Chemical Co. (St. Louis, Mo, USA).

Enzyme source. Ninety-day-old cultures of *L. edodes* commercial heterodikaryon strain no. 852 grown at 22° C within 0.04 mm thick polypropylene plastic bags containing 3.5 kg of a commercial medium consisting of 32% red oak (*Quercus rubra* L.) wood sawdust, 4% millet, 4% wheat bran, and 60% H_2O were provided by Golden Forest (Madison, Wisc, USA).

Enzyme extraction and initial purification. Whole cultures (medium plus fungus) were suspended in distilled deionized H_2O (adjusted to pH 4.0 with HCl [1:2 w/v] at room temperature) and then stirred slowly for 2 h. The crude culture filtrate was filtered through glass wool and the filtrate decolorized by precipitation with 0.9% (v/v) final concentration polyethylenimine. After removing the precipitate by centrifuging at 17000 *g* for 20 min, the filtrate was concentrated 50-fold and diafiltered at 4° C into 50 m*M* sodium acetate (HCl) buffer, pH 4.0, using a Rohm and Haas hollow-fiber concentration unit with a 10000 molecular weight cut-off membrane, having a surface area of 0.093 m^2 (Philadelphia, Pa, USA). The concentrated/diafiltered sample was applied to a 2.5 × 25 cm column packed with 110 ml of DEAE-Trisacryl M anion exchange resin (IBF Biotechnics, Savage, Md, USA) previously equilibrated with the same sodium acetate buffer. The enzyme was eluted, first with a linear 210-ml gradient of 0 to 0.20 *M* NaCl, and then with a linear 165-ml gradient of 0.20 to 0.75 *M* NaCl in the same acetate buffer, using a flow rate of 2 ml/min, collecting 5-ml fractions. Fractions showing activity against glucuronoxylan were pooled and then concentrated and diafiltered into the same sodium acetate buffer using a Schleicher and Schuell collodion bag apparatus with a 10000 molecular weight cut-off membrane (Danvers, Mass, USA).

Preparative isoelectric focusing. Preparative isoelectric focusing was carried out in an LKB Multiphor apparatus as described in LKB application note no. 198 (LKB Produkter, Stockholm, Sweden), replacing the Ultrodex with 40 strips of polyurethane foam (7 × 0.5 × 0.25 cm; total bed volume approximately 60 ml) (Bodhe et al. 1982) placed on gel bond paper (24 × 9 cm). Prior to sample loading, the strips were soaked in LKB ampholine solution, pH 2.4–4.0 (2% w/v). The anodic wick was soaked in L-aspartic acid (25 m*M*) and L-glutamic acid (25 m*M*). The cathodic wick was soaked in ethylenediamine (2 *M*), L-arginine (25 m*M*), and L-lysine (25 m*M*). The sample was then loaded on a center strip and run without prefocusing, as described previously (Mishra et al. 1984). After the run, fractions collected from the foam strips by squeezing were assayed for pH and enzyme activities. Fractions containing xylanase activity were pooled, concentrated, dialyzed in 50 m*M* sodium acetate (HCl) buffer, pH 4.0, and stored at −20° C.

Analytical electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was carried out using a Pharmacia Phast System apparatus and Pharmacia 5% to 25% gradient polyacrylamide gels (Piscataway, NJ, USA) using standard Coomassie blue R250 dye-staining techniques. Ten protein markers were used in the 14200 to 205000 molecular weight range. Analytical isoelectric focusing was carried out on a LKB Multiphor apparatus using Servalyt Precotes gels, pH 3–6 (Serva Fine Biochemicals, Westbury, NY, USA) and Servalyt Coomassie blue stain.

Protein and enzyme assays. Protein content was estimated by the Coomassie G250 dye-binding assay of Spector (1978) using bovine serum albumin Cohn fraction V as standard. Unless otherwise stated, enzyme assays were performed at 40° C in 50 m*M* sodium acetate (HCl) buffer, pH 4.8, using suitably diluted enzyme and the previously described enzyme assay techniques (Leatham 1985); these methods are based on the release of reducing sugars from polysaccharides used at 0.5% final concentration (Somogyi 1952) or *p*-nitrophenol from *p*-nitrophenol-sugar analogs used at 3.33 m*M* (Hägerdahl et al. 1979). The temperature optimum was determined in a heated water bath set to the temperatures specified. The pH optimum was determined in 50 m*M* citric acid (NaOH) buffers adjusted to the pH values specified. Enzyme activities are expressed as international units (μmoles product formed per minute).

Extent of xylan hydrolysis and end-product determinations. Xylan hydrolysis was carried out with 50 mg/ml final xylan concentration. The hydrolysis mixture contained 1.2 units (U) of xylanase activity (based on glucuronoxylan as substrate) from either purified xylanase or crude culture filtrate and 50 m*M* sodium acetate (HCl) buffer, pH 4.8. Hydrolysis was carried out in a 50° C shaking water bath at 25 rpm. Samples were periodically withdrawn and the reducing sugars determined (Somogyi 1952) using L-xylose as standard. Calculations were made based on the total percentage of bonds hydrolyzed assuming that the substrate was pure xylan with one reducing sugar released per 132 molecular weight (anhydro-)monomeric repeat unit. Units are reported as international units (one unit equals one μmole of reducing compound formed per minute). Hydrolysis end-products were determined by paper chromatography using a butanol/pyridine/water (6:4:3) solvent system and a silver nitrate staining procedure (Trevelyn et al. 1950).

Results

Enzyme isolation and estimation of purity

Xylanase activity was purified from the crude culture filtrate by a series of three key separation steps: (1) removal of polyphenol-like compounds, (2) anion exchange chromatography, and (3) isoelectric focusing. The removal of the interfering brown polyphenols by precipitation with dilute polyethylenimine solution solved a troublesome technical problem. Without removal, the polyphenols bound tightly to the anion exchange resin, hindered separation, and after only a few separations, rendered the resin useless.

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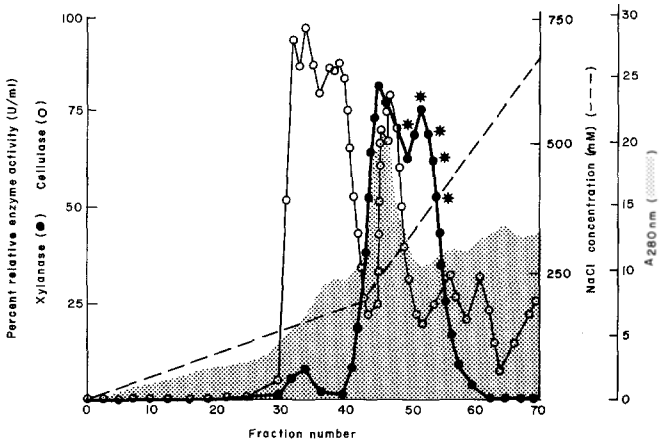

Fig. 1. Anion exchange chromatography of the crude culture filtrate on DEAE-resin eluting with a NaCl gradient. The fractions collected showed activity against glucuronoxylan (●) and acid-swollen cellulose (units [U]/ml) (○); NaCl concentration (– – – –); and absorption at 280 nm ($A_{280\,nm}$; *shaded area*). *Asterisks* denote the fractions collected for further purification

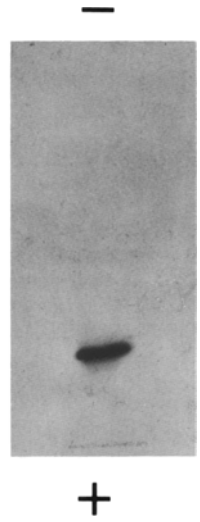

Fig. 3. Determination of xylanase purity by analytical gel isoelectric focusing

Separation by anion exchange chromatography gave two major peaks of apparent xylanase activity (Fig. 1). However, the xylanase activity in the first peak was strongly overestimated due to the presence of a non-specific cellulase (Fig. 1). Therefore, only fractions in the second peak were pooled and purified further. Preparative isoelectric focusing yielded a single xylanase activity peak, distinct from arabinosidase and xylosidase peaks (Fig. 2).

The final xylanase preparation was obtained by diafiltering and concentrating the isoelectric fractions with the highest xylanase and lowest possible oligosaccharidase activities (nos. 17, 18, and 19; Fig. 2). The preparation was found free from any major contaminating proteins by both SDS-PAGE and analytical gel isoelectric focusing using Coomassie blue dye-staining (Fig. 3). Overall, the xylanase was purified 8.9-fold (Table 1).

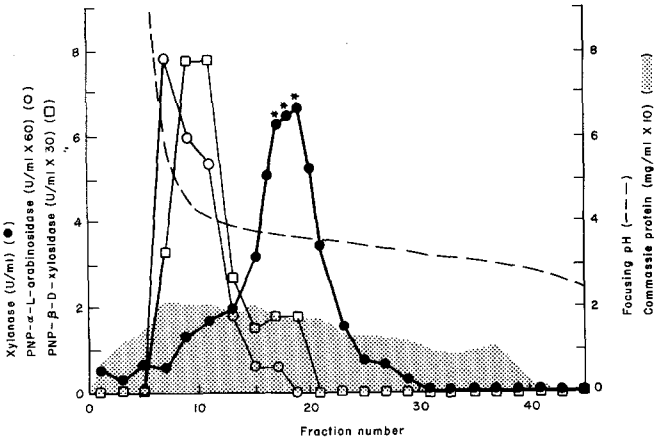

Fig. 2. Preparative isoelectric focusing of the xylanase peak collected from anion exchange chromatography. The fractions collected showed: activity against glucuronoxylan (U/ml) (●), *p*-nitrophenol-(PNP)-α-L-arabinofuranoside (U/ml × 60) (○), and PNP-β-D-xylopyranoside (U/ml × 30) (□); isoelectric focusing pH (– – – –); and Coomassie protein *(shaded area). Asterisks* denote the fractions used to produce the purified xylanase

Enzyme characteristics

Several characteristics of the purified xylanase were determined. The enzyme exhibited a single polypeptide band with an apparent molecular weight of 41 000 (on SDS gels) and a pI value of 3.6. With aspen glucuronoxylan as substrate, the purified xylanase showed a pH optimum between 4.5 and 5.0 (Fig. 4) and a temperature optimum of 60° C (Fig. 5). The enzyme showed excellent stability: essentially no activity was lost from solutions containing purified enzyme that were repeatedly freeze-thawed or frozen for several weeks in 50 m*M* sodium acetate buffer, pH 4.0, and little activity was lost at 40° C during 48-h-long xylan hydrolysis assays.

The xylanase showed good activity and marked specificity towards β-(1,4)-D-xylans. At pH 4.8 and 40° C, a specific activity of 310 U/mg was obtained with either aspen glucuronoxylan (4-*o*-methyl-α-(1,2)-D-glucuronic acid branched) or oat spelt arabinoxylan (α-(1,3)-L-arabinofuranose branched). Under these conditions the substrate binding constant (K_m) for aspen glucuronoxylan was determined to be 0.66 mg/ml using a Lineweaver-Burk plot.

In contrast, the xylanase showed greatly reduced activity against laminarin (β-(1,3)-D-glucose branched β-(1,6)-D-glucan; 53 U/mg) and little activity (9 U/mg) against either pachyman (β-(1,3)-D-glucan) or pustulan (β-(1,6)-D-glucan). The xylanase had no detectable cellulase activity against acid-swollen cellulose or carboxymethylcellulose and no detectable oligosaccharidase activity against salicin or *p*-nitrophenol-linked sugar analogs including the β-D-cellobioside, β-D-xylopyranoside, β-D-glucopyranoside, β-D-galactopyranoside, β-D-mannopyranoside, and α-L-arabinofuranoside.

Table 1. Purification table for the xylanase of *Lentinula edodes*

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor
Crude culture filtrate	1834	65800	35	1.0
Polyethylenimine decolorization	280	23800	82	2.3
Anion exchange chromatography	24	1512	63	1.8
Preparative isoelectric focusing	0.6	186	310	8.9

U = units

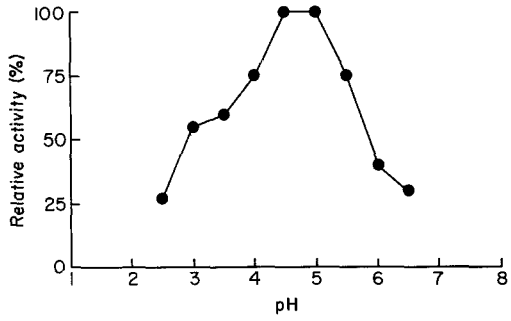

Fig. 4. Determination of xylanase pH optimum with glucuronoxylan as substrate

The xylanase was capable of extensive xylan hydrolysis. When using 50 mg/ml (5%) of either glucuronoxylan or arabinoxylan at 50° C, 1.2 U/ml of purified enzyme hydrolyzed 40% or 60% of the hydrolyzable bonds present (see Materials and methods) within 24 h, respectively (Fig. 6). However, the enzyme was less capable of xylan hydrolysis than was the crude filtrate. When added at an equivalent activity level (based on xylanase activity against aspen glucuronoxylan) and tested under the same conditions, the crude culture filtrate hydrolyzed 78% or 86% of the bonds present in glucuronoxylan or arabinoxylan, respectively.

Examination of hydrolysis end-products suggested that the xylanase was probably a non-debranching endoxylanase (Fig. 7). Major identifiable products resulting from glucuronoxylan hydrolysis by the purified enzyme consisted of a mixture of predominantly xylobiose, larger xylooligosaccharides (including X_3 and X_4), and xylose (X_1), but no glucuronic acid. The major identifiable products from arabinoxylan showed an analogous pattern, except that spots for the smallest arabinose-linked xylooligosaccharides appeared to be present including arabinoxylobiose (X_2A) and arabinoxylotriose (X_3A), and no arabinose was produced. In contrast to the purified xylanase, crude culture filtrate produced a distinct arabinose or glucuronic acid spot from arabinoxylan or glucuronoxylan, respectively (Fig. 7). The non-debranching nature of the purified enzyme was again indicated using a 400-fold increase in the ratio of enzyme to xylan substrate, i.e. 10 U enzyme with 1 mg xylan/ml reaction mixture in contrast to the previously tested 1.2 U with 50 mg. Again, neither glucuronic acid or arabinose were produced.

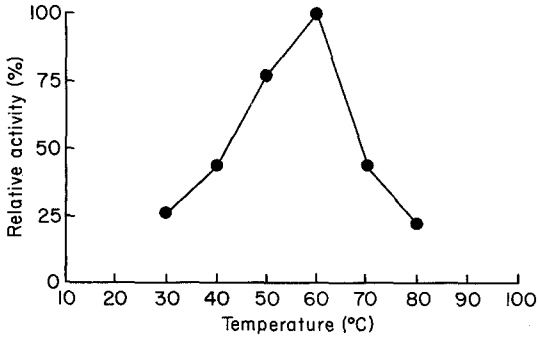

Fig. 5. Determination of xylanase temperature optimum with glucuronoxylan as substrate

Discussion

In studying a major extracellular xylanase from *L. edodes* cultures grown on a commercial solid wood medium, the following observations were made: (1) the enzyme can be purified from a complex crude culture filtrate; (2) it has an apparent molecular weight of 41000 (on SDS gels) and an isoelectric point of 3.6; (3) it appears to be a non-debranching endo-type xylanase markedly specific for β-(1,4)-D-xylans, which lacks both cellulase activity and oligosaccharidase activity against salicin and *p*-nitrophenol (PNP)-sugar analogs; (4) the

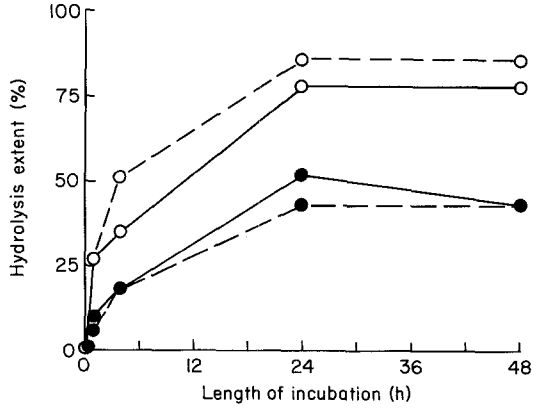

Fig. 6. Determination of the extent of glucuronoxylan (——) or arabinoxylan (- - - -) hydrolysis after different lengths of incubation by purified xylanase (●) or crude enzyme filtrate (○) added at equivalent levels of xylanase activity

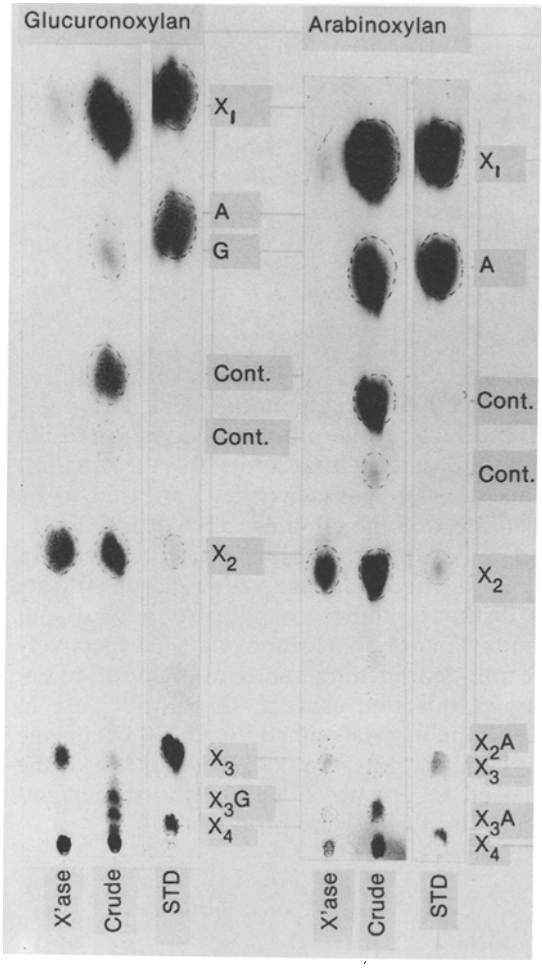

Fig. 7. Determination of the end-products of arabinoxylan and glucuronoxylan hydrolysis produced by purified xylanase *(X'ase)* or crude culture filtrate *(Crude)* after 48 h incubation (see Fig. 6). Spot positions are shown for xylose *(X_1)*, arabinose *(A)*, glucuronic acid *(G)*, xylobiose *(X_2)*, xylotriose *(X_3)*, and xylotetrose *(X_4)*. Also shown are spots for apparent arabinoxylobiose *(X_2A)*, arabinoxylotriose *(X_3A)*, and glucuronoxylotriose *(X_3G)*. Note that the crude culture filtrate was contaminated with glucose *(Cont.)* and an unknown sugar *(Cont.)*

pH and temperature optima of the enzyme for glucuronoxylan degradation are near 4.5–5.0 and 60° C, respectively; (5) the enzyme is capable of extensive xylan saccharification (40%–60% under the conditions tested); and (6) the complete xylanolytic system of *L. edodes* in crude culture filtrates is capable of even more extensive xylan saccharification than is the purified enzyme. To our knowledge, this is the first report of the purification and characterization of a xylanase from a commercially important lignocellulose-degrading microorganism growing on a solid wood medium. The source of the enzyme is important; it suggests that the enzyme is likely to have direct relevance in commercial bioconversion of wood.

The complexity of the crude culture filtrate used here caused compromises in both xylanase purification and assay. The actual xylanase activity present was overestimated in crude preparations because of the presence of synergistic enzymes. Moreover, the total xylanase activity dropped rapidly during purification primarily because of the removal of synergistic enzymes. We also found it necessary to accept to lower yield than would have been possible by only taking the dominant peak fractions. This allowed expedient recovery of the xylanase and helped to avoid contamination with other enzymes.

The xylanase obtained from *L. edodes* should be compared with those characterized from other microorganisms. As is typical for most xylanases, the enzyme produced by *L. edodes* appears to be a non-debranching endo-β-(1,4)-D-xylanase (e.g. *Schizophyllum commune:* Paice et al. 1978; *Cryptococcus albidus:* Biely et al. 1980; *Bacillus circulans:* Uchino and Nakane 1981) with a pH optimum and molecular weight within the most commonly observed ranges of 4.0–6.0 and 12000–45000 (on SDS gels), respectively.

However, some characteristics of the *L. edodes* enzyme are less typical for fungal endo-xylanases and may be useful to industry. Perhaps the most notable is the high specificity, for example lack of significant cellulase activity (Mishra et al. 1984; Rouau and Odier 1986; Wong et al. 1988). The *L. edodes* enzyme also has a high relative temperature optimum, specific activity, and capacity for xylan hydrolysis and has a lower than average pI value. Its temperature optimum and K_m for glucuronoxylan were identical to those reported for the high-affinity, high-temperature optimum "xylanase I" of *Trichoderma koningii* (Wood and McCrae 1986). The specificity and temperature optimum appear similar to that of the xylanase of *Streptomyces lividans* (Morosoli et al. 1986).

In contrast, the other fungal xylanases characterized so far typically have lower temperature optima (40°–50° C; e.g. *Aspergillus niger,* Fournier et al. 1985), higher (weaker affinity) K_m values (1–3 mg/ml; e.g. *T. koningii* "xylanase II": Wood and McCrae 1986), lower hydrolysis capacities (25%–35%; e.g. *Neurospora crassa:* Mishra et al. 1984; *T. koningii:* Wood and McCrae 1986), and higher pI values (e.g. 8.0 to 9.5 for *T. viride:* Dekker 1985; 7.2–7.3 for *T. koningii:* Wood and McCrae 1986; 4.5, 6.7, 8.6 and 9.0 for *A. niger:* Fournier et al. 1985; and 9.4 and 9.5 for *Trichoderma harzianum:* Tan et al. 1985). Finally, the hydrolytic capacity of the crude culture filtrate of *L. edodes* was high compared to that reported for other crude xylanase preparations (e.g., 40% for *Penicillium funiculosum,* Rao et al. 1986).

The information reported here should facilitate the improvement of industrial strains of *L. edodes* (Lu et al. 1989) and development of applications for isolated xylanolytic enzymes. Additional research is needed to isolate and characterize the other major xylanolytic enzymes extractable from cultures of *L. edodes* grown on solid substrates. Those likely to be present in crude culture filtrates include a debranching α-(1,3)-L-arabinofuranosidase, debranching α-(1,2)-D-glucuronidase (Puls et al. 1987; Ishihara and Shimizu 1988), and β-D-xylosidase (Reilly 1981; Wong et al. 1988) as well as the deacetylase (Biely et al. 1985a, b) and demethylase required for the complete hydrolysis of native xylans

(Reilly 1981; Biely 1985; Dekker 1985; Poutanen et al. 1987).

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