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Agricultural Residues as Source for Production of Hemicellulases from *Humicola grisea* var. *thermoidea*

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ABSTRACT

The filamentous fungus *Humicola grisea* var. *thermoidea* produces hemicellulose-degrading enzyme activities when grown in the presence of different carbon sources, including wheat bran, oat spelt xylan, cellulose (avicel), oat bran, banana stem and coffee spent-ground. The best yield of β-xylanase, β-mannanase and α-arabinofuranosidase activities was given by the crude extract sample from a medium containing coffee spent-ground as the carbon source. The previous crude extract preparation was submitted to ultrafiltration in an Amicon system fitted with a 10 kDa-cut-off membrane. A considerable amount of β-xylanase, β-mannanase and α-arabinofuranosidase activities was found in the retentate and used for subsequent characterization. β-Mannanase, β-xylanase and α-arabinofuranosidase showed highest activity at 50, 55 and 80°C, respectively. β-Mannanase and α-arabinofuranosidase activities were stable at 50°C. However, β-xylanase was less stable. A purified β-xylanase (Xyn III), obtained through growth of the filamentous fungus *H. grisea* var. *thermoidea* in liquid medium containing banana stem as carbon source, was characterized with regard to some enzymatic properties and its action on xylan. The enzyme showed best activity at pH 7.0 and 55°C and half life of 20 min at 60°C. Xyn III exhibited preferential activity towards xylan. The determination of kinetic parameters showed that the enzyme had more affinity against insoluble arabinoxylan as substrate. DTT and DTP activated Xyn III. In contrast, NBS, XIP-1, EDC and iodoacetamide inhibited the enzyme activity.

Keywords: banana stem, coffee spent-ground, mannanase, xylanase

INTRODUCTION

The banana stem, grain stalk that supports the banana fruits readily available in tropical and subtropical countries, is normally discarded after the fruit harvesting, either in the "packing houses" or in the delivering centers, where it is considered a residue due to the great volume generated, contributing to serious environmental problems (Medeiros *et al.* 2000; Salles *et al.* 2005). It is a low-cost residual component where holocellulose and lignin account for as much as 33% and 8.67% of the dry weight, respectively. Coffee spent-ground is an important agro-residual residue in coffee-producing countries (Leifa *et al.* 2001). It is obtained during the processing of raw coffee powder to prepare instant coffee and also contains lignin, hemicellulose and cellulose in its composition. For these reasons, banana stem and coffee spent-ground can be considered alternative carbon sources for enzyme production.

Xylan and mannan are the major constituent of the hemicelluloses with linear or branched backbone of β-1,4-linked xylopyranose and β-1,4-linked mannopyranose units, respectively (Polizeli *et al.* 2005; Salles *et al.* 2005; Wu *et al*. 2008). Hemicellulases are produced by a variety of fungus species (Medeiros *et al.* 2000), including *Humicola grisea* var. *thermoidea*. A combination of main- and side-chain enzymes is necessary to effect the complete hydrolysis of xylan and mannan. β-Xylanase (EC 3.2.1.8) and β-mannanase (EC 3.2.1.78) are main-chain enzymes that cleaves internal bonds at random or at specific position, releasing long and short oligosaccharides (Franco *et al.* 2004; Salles *et al.* 2005). Additional enzymes such as α-arabinofuranosidase are required to remove side-chain substituents (Subramaniyan and Prema 2002; Franco *et al.* 2004).

There is a great interest in the use of hemicellulase as alternative approach in pulp bleaching (Medeiros *et al.* 2002; Esteghlalian *et al*. 2008). The most significant effects of enzymes on pulp bleaching are reduction in the requirement for elemental chlorine and decrease in the amount of organochlorine compounds in bleach plant effluents (Medeiros *et al.* 2002). In previous articles (Salles *et al.* 2005; Medeiros *et al.* 2007), we reported the purification of a novel xylanase (Xyn III) from *H. grisea* var. *thermoidea*, after growth in medium containing banana stem, by using gel filtration and ion-exchange as chromatographic procedures and its effect on eucalyptus pulp bleaching through spectrophotometric, scanning electron microscopy and atomic force microscopy techniques. Xyn III caused morphological modifications in the surface of cellulose fibres and significant changes in the topography, texture and elasticity of kraft pulp when submitted to scanning electron and atomic force microscopy procedures, respectively. In this present work, we describe different aspects of the production of hemicellulase activities from *H. grisea* var. *thermoidea:* the growth in liquid media containing different carbon sources and characterization of a purified xylanase (Xyn III).

MATERIALS AND METHODS

Chemicals

Oat spelt xylan, locust bean gum (galactomannan), carboxymethyl cellulose (CMC), *p*-nitrophenyl-β-D-glucopyranoside (PNPG), *p*-nitrophenyl-β-D-xylopyranoside (PNPX), *p*-nitrophenyl-α-D-arabi-nofuranoside (PNPA), *p*-nitrophenyl-β-D-manopyranoside (PNPM), pectin from citrus fruits, dithiothreitol (DTT), 2,2'-Dithiodipyridine (DTP), N-bromosuccinimide (NBS), 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC), ioacetamide, avicel and diethyl pyrocarbonate (DEPC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). XIP-I (xylanase inhibitor protein) and banana stem were kindly provided by Nathalie Juge (Norwich Research Park, UK) and Francides Gomes da Silva Jr. (Esalq/USP, Brazil), respectively. All other chemicals were analytical grade reagents.

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Table 1 Summary of the purification of Xyn III from *H. grisea* var. *thermoidea.*

Purification step	Total protein (mg)	Total activity (IU)	Specific activity (IU/mg)	Purification (-fold)	Yield (%)
Crude extract	59.00	1275.0	21.6	1	100
Sephadex G-50	0.59	155.8	264.1	12.2	12.23
Sephadex G-75	0.02	18.0	900	41.6	1.41
DEAE-Sepharose	0.002	42.5	21250	938.8	3.33

Microorganism

For production of hemicellulase activities (xylanase, mannanase and arabinofuranosidase), *H. grisea* var. *thermoidea* was cultured at 42°C for 8 days with rotary shaking (100 rpm) in liquid-state media containing wheat bran, oat spelt xylan, cellulose (avicel), oat bran, banana stem or coffee spent-ground as the carbon sources. Flasks containing the carbon source (1%) and supplemented medium (0.7% KH_2PO_4, 0.2% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% $(NH_4)_2SO_4$, and 0.06% yeast extract at pH 7.0) were inoculated with spore suspension (1.0×10^7 mL^{-1}) from a routine subculture. After the growth procedure, the crude extract was filtered through filter paper and stored at 5°C for subsequent use as source of hemicellulase.

Enzyme purification

Xyn III was purified to apparent homogeneity by ultrafiltration, followed by gel filtration and ion-exchange chromatographies, respectively (Salles *et al.* 2005).

Enzyme assays

Oat spelt xylan was used as the substrate for xylanase activity. Xylan (1.0 g) was previously treated with 20 mL of 1.0 M NaOH and 20 mL of 1.0 M HCl, and the volume was brought to 100 mL with 50 mM sodium acetate buffer pH 5.0, followed by stirring for 1 h at 25°C. The insoluble xylan was removed by centrifugation (12,000 × *g*) for 15 min at 7°C and the soluble fraction was used for xylanase assay. Xylanase activity was routinely determined by mixing 50 µL of enzyme solution with 100 µL of soluble oat spelt xylan in 50 mM sodium acetate buffer, pH 5.0, at 50°C for 30 min. The release of reducing sugar was measured using the dinitrosalicylic reagent method (Miller 1959). Xylose was used as the standard. Enzyme activities were expressed as µmol product formed min^{-1} mL^{-1} of enzyme solution, i.e., as IU mL^{-1}. Cellulase, β-xylosidase, β-glucosidase, α-arabinofuranosidase, β-mannosidase, β-mannanase and pectinase assays were carried out as described elsewhere (Mandels *et al.* 1976; Filho 1996; Ximenes *et al.* 1996; Ferreira and Filho 2004). Protein concentration was measured by the method of Bradford (1976), using bovine serum albumin as standard. For the kinetic experiments, soluble and insoluble samples from oat spelt xylan were used as substrates in a concentration range of 3.33-16.3 mg/mL and 1.0-10 mg/mL, respectively. The substrates were prepared as described by Filho *et al.* (1993). K_m and V_{max} values were estimated from the Michaelis-Menten equation with a non-linear regression data analysis program (Leatherbarrow 1987). The effect of temperature on the enzyme activities was determined by performing the standard xylanase assay at a temperature range of 30 to 80°C. The effect of pH was carried out at 50°C using the following buffers: 50 mM sodium acetate buffer (3.0-6.0); 50 mM sodium phosphate (6.0-7.5); 50 mM Tris-HCl (7.5-8.5). All buffer systems were adjusted with the same ionic strength with NaCl. Temperature stability was determined by pre-incubating an enzyme sample at 60°C. At various time periods, aliquots were withdrawn and the residual activity was measured under standard conditions. The effect of some reagents at 10 mM (NBS, DEPC, EDC, DTP and iodoacetamide), 20 mM (DTT) and 100 µg/mL (XIP-1) concentrations on the Xyn III activity was determined by performing the assay under the same conditions as described above. All activity values were repeated at least three times. The standard deviation for enzyme assays was less than ±20% of the mean.

SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) using 12% gel. Protein bands in the gel were visualized by the silver staining method (Blum *et al.* 1987).

RESULTS AND DISCUSSION

Enzyme production

H. grisea var. *thermoidea* was screened for the production of hemicelulase activity (β-xylanase, β-mannanase and α-arabinofuranosidase) when grown by liquid-state cultivation on xylan, wheat bran, oat bran, cellulose or coffee spent-ground as the carbon sources. Although fungal growth was abundant on all carbon sources, there were differences in the production of hemicellulase (result not shown). Coffee spent-ground was selected as the best substrate for production of hemicellulase activity, followed by wheat bran. The hemicellulase preparation obtained from the culture medium containing coffee spent-ground as the substrate was then submitted to ultrafiltration. Most of the hemicelulase activities were retained by the membrane. However, a low xylanase activity was found to be present in the ultrafiltrate. Cellulase and pectinase activities were also found in the concentrate. The hemicellulase activities from concentrate were partially characterized. β-Mannanase, β-xylanase and α-arabinofuranosidase showed highest activity at 50, 55 and 80°C, respectively. β-Mannanase and α-arabinofuranosidase activities were stable at 50°C with a half-life above 24 h. However, β-xylanase was less stable, showing a half-life below 24 h.

Characterization of Xyn III

Xyn III purification procedure was previously described before (Salles *et al.* 2005). The protein exhibited a monomeric structure and migrated as approximately 25 kDa band on SDS-PAGE stained with silver nitrate (Salles *et al.* 2005). The purification steps are summarized in **Table 1**. The recovery of Xyn III was very low (3.33%). The low yield value could be credited to the fact that Xyn III was separated from other xylan-degrading enzymes during the purification steps. For example, the gel filtration chromatography profile on Sephadex G-50 showed three peaks of xylanase activity (result not shown). Since that xylanases act synergistically on xylan degradation, the yield and fold purification were underestimated (Cardoso and Filho 2003). Some properties of the purified Xyn III are discussed below and compared to xylanases (forms 1and 2 and X2) from *H. grisea* var. *thermoidea* (Monti *et al.* 1991; Lucena-Neto and Filho 2004) and some other fungal species. The molecular mass of Xyn III (25 kDa), described elsewhere (Salles *et al.* 2005), was within the range for xylanases belonging to family G/11 (Salles *et al.* 2000). It differed from the ones purified from crude extract samples of the same fungus, which were 13, 23, and 29 kDa (Monti *et al.* 1991; Lucena-Neto and Filho 2004). The molecular mass of Xyn III was higher than the values determined for XynC (22 kDa) and XynII (22.16 kDa) from *Penicillium capsulatum* and *Acrophialophora nainiana*, respectively (Salles *et al.* 2000; Ryan *et al.* 2003).

The enzyme exhibited a maximal activity at 55°C, however, with a rapid decline at 70°C (**Fig. 1**). This result was

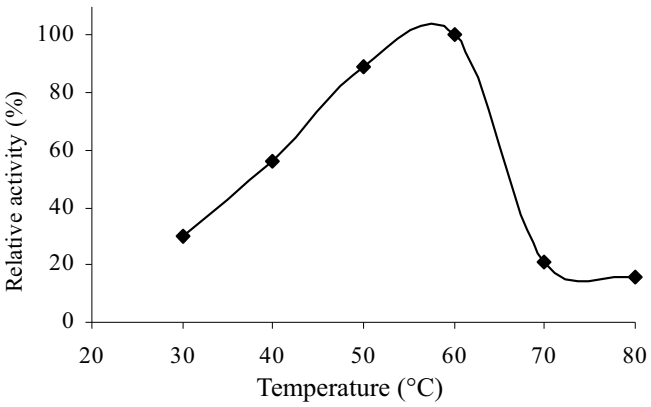

Fig. 1 The effect of temperature on Xyn III activity.

Table 2 The kinetic parameters of Xyn III.

Kinetic parameter	Arabinoxylan	
	Soluble	Insoluble
K_m (mg mL^{-1})	7.4	1.22
V_{max} (IU)	6.7	6.2
K_{cat} (min^{-1})	6.1	6.23
$K_{cat}Km^{-1}$ (min^{-1} mg^{-1} mL)	0.8	5.1

Table 3 The Effect of some reagents on the activity of Xyn III.

Reagent	Relative activity (%)
Control	100
DTT	305
NBS	22
Iodoacetamide	75
EDC	84
DTP	166
DEPC	94
XIP I	50

less than those of xylanases forms 1 and 2 and similar to xylanase X2. It is noteworthy that Xyn III was subjected to a 30-min assay, while xylanase forms 1 and 2 had an incubation time of only 15 min. Like xylanases form 2 and from *Termitomyces* sp. (Faulet *et al.* 2006), the enzyme was quite stable at 50°C. It also showed a half-life of 20 min at 60°C. In opposite to xylanase form 2, Xyn III was most active at pH 7.0, with a steady and gradual decline of its activity. At pH values of 6.5, 7.5, 8.0 and 8.5, the activity was 88, 94, 75 and 54% of the optimum value, respectively.

Xyn III was devoid of measurable cellulase (FPase, CMCase and, β-glucosidase), α-arabinofuranosidase, β-xylosidase, pectinase, mannanase and β-mannosidase activities. Therefore, the substrate specificity of Xyn III seems to be restricted to xylan. This result gives more support to the hypothesis that Xyn III belongs to the glycosyl family G/11. Besides, the specificity of Xyn III is an important parameter for its use in pulp bleaching, whereas in this process the enzyme has to be cellulase free. Xylanase form 2 and X2 were also cellulase free enzymes, while β-glucosidase activity was detected for xylanase form 1. Xylanase from *Termitomyces* sp was active against CMC (Faulet *et al.* 2006).

Kinetic measurements at 50°C with insoluble and soluble oat spelt xylans were according the Michaelis-Menten model. A significant difference in apparent Km values for soluble and insoluble oat spelt xylans was observed (**Table 2**). The Km value for soluble xylan was much higher than the insoluble one. This might suggests a steric hindrance due the presence of side-chains groups in soluble xylan (Silva *et al.* 1999). It may be noted that K_{cat} and $K_{cat}Km^{-1}$ (catalytic specificity) were highest with insoluble oat spelt xylan. Compared with X2, Xyn III showed higher affinity for the soluble and insoluble forms of oat spelt xylan. The apparent K_m value for insoluble oat spelt xylan (1.22 mg mL^{-1}) was much lower than the K_m values of the xylanases from *A. nainiana* and *Thermomyces lanuginosus* CBS 288.54 (Cardoso and Filho 2003; Li *et al.* 2005).

The involvement of some modifying agents on Xyn III activity was investigated (**Table 3**). Xyn III was activated by DTT and DTP and inhibited by NBS, iodoacetamide, EDC and XIP-I. The enzyme activation by DTT and DTP suggests an influence of L-cysteine in the catalysis of xylan. An explanation for this would be an involvement of L-cysteine in hydrogen-bonding with the substrate, enzyme folding and the formation of the covalent glycosyl-substrate intermediate (Filho *et al.* 1993; Ferreira and Filho 2004). Besides, xylanase activation by DTT indicates that the reduction of disulphide bridge(s), probably oxidized during enzyme extraction and purification procedures, restores the native enzyme conformation (Filho *et al.* 1993; Franco and Ferreira 2004). A similar result was found for XynC from *P. capsulatum* (Ryan *et al.* 2003). Treatment with iodoacetamide had negative effect on Xyn III activity. Evidence for involvement of the L-tryptophan residue in binding of Xyn III to xylan was given by the high inhibition of Xyn III by NBS, a strong oxidizing agent of L-tryptophan (Filho *et al.* 1993). This result corroborates the findings on the XynC and XynIII from *P. capsulatum* and *A. nainiana*, respectively (Salles *et al.* 2000; Cardoso and Filho 2003). Small inactivation by DEPC and EDC corroborates evidences for the involvement of histidine and carboxyl groups in binding or catalysis, respectively. Xyn III was also inactivated by XIP-I, an extracellular protein from wheat capable of inhibiting fungal endo-(1,4)-β-endoxylanases (Juge *et al.* 2004).

CONCLUSIONS

In conclusion, *H. grisea* var. *thermoidea* produces hemicellulase activities when grown in agricultural residues. In comparison with other β-xylanases produced by the same fungus, Xyn III has different properties, such as pH and temperature optima, kinetic parameters, thermostability and molecular mass.

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REFERENCES

Blum H, Beier H, Gross B (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8**, 93-99

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry* **72**, 248-254

Cardoso OAV, Filho EXF (2003) Purification and characterization of a novel cellulase-free xylanase from *Acrophialophora nainiana*. *FEMS Microbiology Letters* **223**, 309-314

Esteghlalian AR, Kazaoka MM, Lowery BA, Varvak A, Hancock B, Woodward T, Turner JO, Blum DL, Weiner D, Hazlewood GP (2008) Prebleaching of softwood and hardwood pulps by a high performance xylanase belonging to a novel clade of glycosyl hydrolase family 11. *Enzyme and Microbial Technology* **42**, 395-403

Faulet BM, Niamké S, Gonnety JT, Kouamé LP (2006) Purification and biochemical properties of a new thermostable xylanase from symbiotic fungus, *Termitomyces* sp. *African Journal of Biotechnology* **5**, 273-282

Ferreira HMF, Filho EXF (2004) Purification and characterization of a β-mannanase from *Trichoderma harzianum* strain T4. *Carbohydrate Polymers* **57**, 23-29

Filho EXF, Puls J, Coughlan MP (1993) Biochemical characteristics of two endo-β-xylanases produced by *Penicillium capsulatum*. *Journal of Industrial Microbiology* **11**, 171-180

Filho EXF (1996) Purification and characterization of a β-glucosidase from solid-state cultures of *Humicola grisea* var. *thermoidea*. *Canadian Journal of Microbiology* **42**, 1-5

Franco PF, Ferreira HM, Filho EXF (2004) Production and characterization of hemicellulase activities from *Trichoderma harzianum* strain T4. *Biotechnology and Applied Biochemistry* **39**, 1-5

Juge N, Payan F, Williamson G (2004) XIP-I, a xylanase inhibitor protein from wheat: a novel protein function. *Biochimica et Biophysica Acta* **1696**,

203-211

Laemmli UK (1970) Cleavage of structural proteins during the assembly ot he head of bacteriophage T4. *Nature* **227**, 680-685

Leatherbarrow RJ (1987) *Enzfitter Manual*, Biosoft, London, pp 13-42

Leifa F, Pandey A, Soccol CR (2001) Production of *Flammulina velutipes* on coffee husk and coffee-spent-ground. *Brazilian Archives of Biology and Technology* **44**, 205-212

Li XT, Jiang ZQ, Li LT, Yang SQ, Feng WY, Fan JY, Kusakabe I (2005) Characterization of a cellulase-free, neutral xylanase from *Thermomyces lanuginosus* CBS 288.54 and its biobleaching effect on wheat straw pulp. *Enzyme and Microbial Technology* **96**, 1370-1379

Lucena-Neto AS, Filho EXF (2004) Purification and characterization of a new xylanase from *Humicola grisea* var. *thermoidea*. *Brazilian Journal of Microbiology* **35**, 86-90

Mandels M, Andreotti R, Roche C (1976) Measurement of saccharifying cellulase. *Biotechnology and Bioengineering Symposium* **16**, 21-33

Medeiros RG, Soffner MLAP, Thomé JA, Cacais AOG, Estelles RS, Salles BC, Ferreira HM, Lucena Neto SA, Silva Jr. FG, Filho EXF (2000) The production of hemicellulases by aerobic fungi on medium containing residues of banana plant as substrate. *Biotechnology Progress* **16**, 522-524

Medeiros RG, Silva Jr. FG, Salles BC, Estelles RS, Filho EXF (2002) The performance of fungal xylan-degrading enzyme preparations in elemental chlorine-free bleaching for *Eucalyptus* pulp. *Journal of Industrial Microbiology and Biotechnology* **28**, 204-206

Medeiros RG, Silva LP, Azevedo R B, Silva Jr. FG, Filho EXF (2007) The use of atomic force microscopy as a tool to study the effect of a xylanase from *Humicola grisea* var. *thermoidea* in kraft pulp bleaching. *Enzyme and Microbial Technology* 723-731

Miller GL (1959) Use of dinitrosalicylic acid reagent for the determination of reducing sugar. *Analytical Chemistry* **31**, 426-428

Monti R, Terenzi HF, Jorge JA (1991) Purification and characterization of an extracellular xylanase from the thermophilic fungus *Humicola grisea* var. *thermoidea. Canadian Journal of Microbiology* **37**, 675-681

Polizeli MLT, Rizzatti ACS, Monti R, Terenzi HF, Jorge JA, Amorim DS (2005) Xylanases from fungi: properties and industrial applications. *Applied Microbiology and Biotechnology* **67**, 577-591

Ryan SE, Nolan K, Thompson R, Gubitz GM, Savage AV, Tuohy MG (2003) Purification and characterization of a new low molecular weight endoxylanase from *Penicillium capsulatum*. *Enzyme and Microbial Technology* **33**, 775-785

Salles BC, Cunha RB, Fontes W, Sousa MV, Filho EXF (2000) Purification and characterization of a new xylanase from *Acrophialophora nainiana*. *Journal of Biotechnology* **81**, 199-204

Salles BC, Medeiros RG, Báo SN, Silva Jr. FG, Filho EXF (2005) Effect of cellulase-free xylanases from *Acrophialophora nainiana* and *Humicola grisea* var. *thermoidea* on eucalyptus kraft pulp. *Process Biochemistry* **40**, 343-349

Silva CHC, Puls J, Sousa MV, Filho EXF (1999) Purification and characterization of a low molecular weight xylanase from solid-state cultures of *Aspergillus fumigatus* Fresenius. *Brazilian Journal of Microbiology* **30**, 114-119

Subramaniyan S, Prema P (2002) Biotechnology of microbial xylanases: enzymology, molecular biology, and applications. *Critical Reviews in Biotechnology* **22**, 33-64

Wu H, Prei J, Wu G, Shao W (2008) Overexpression of GH10 endoxylanase XynB from *Thermotoga maritima* in Escherichia coli by a novel vector with potential for industrial application. *Enzyme and Microbial Technology* **42**, 230-234

Ximenes FA, Silveira FQP, Filho EXF (1996) Production of β-xylosidase activity by *Trichoderma harzianum* strains. *Current Microbiology* **33**, 71-77