PERGAMON

Soil Biology and Biochemistry 31 (1999) 1735–1740

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Activity and partial characterisation of xylanolytic enzymes in the earthworm *Eisenia andrei* fed on organic wastes

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Accepted 25 May 1999

Abstract

This study demonstrates the occurrence of active xylanolytic enzymes in extracts from *Eisenia andrei*, Bouché 1972. Xylanase was the most active enzyme (2.06 U g^{-1}), followed by acetyl xylan esterase (0.55 U g^{-1}). Lower activities showed β-D-glucuronidase (0.17 U g^{-1}), α-L-arabinosidase (0.04 U g^{-1}) and β-xylosidase (0.03 U g^{-1}). All of the xylanolytic enzymes displayed higher total activity when the earthworms were fed on a xylan-containing medium. Xylanase, α-L-arabinosidase and acetyl xylan esterase showed maximal activity at neutral pH. In contrast, β-xylosidase and β-glucuronidase displayed maximal activity at pH 3.0 and 4.0, respectively. All enzymes displayed a high activity within the physiological temperature range 35 to 40°C. K_m and V_{max} values of xylanolytic enzymes were also estimated. Kinetic parameters for xylanase were determined in extracts of earthworms fed on standard medium ($K_m = 6.91 \pm 1.120$ mg ml^{-1}; $V_{max} = 0.05 \pm 0.002$ U mg^{-1}), and in extracts of earthworms fed on a medium containing xylan ($K_m = 5.60 \pm 1.070$ mg ml^{-1}; $V_{max} = 0.12 \pm 0.006$ U mg^{-1}).

Keywords: Xylanolytic enzymes; Earthworm; *Eisenia andrei*; Paper-pulp mill sludge

1. Introduction

Xylan is the main component of plant hemicellulose and, after cellulose, the second most abundant renewable polysaccharide in nature. It is structurally complex, and its total hydrolysis to xylose is achieved by the synergistic action of various exo-hydrolases (e.g. acetyl xylan esterase, α-L-arabinosidase, β-xylosidase, β-glucuronidase) and an endo-xylanase (Biely, 1985; Bachmann and McCarthy, 1991). While considerable effort has been focused on the β-endo-1,4-xylanase (E.C. 3.2.1.8) and on β-D-xylosidase (E.C. 3.2.1.37), there is much more limited information about other hemicellulases responsible for cleavage of side groups: α-L-arabinosidase (E.C. 3.2.1.55), β-D-glucuronidase (E.C. 3.2.1.31) and acetyl xylan esterase (E.C. 3.1.1.6). However, the lack of just one of these enzymes may result in the accumulation of oligomeric degradation products (Puls et al., 1988; Bachmann and McCarthy, 1991).

It has long been assumed that most invertebrates do not posses the enzymatic complement to digest polysaccharides, but now the opposite is often shown for different groups of soil fauna, enabling us redefine species' diets and therefore their ecological function. Some authors state that some earthworms have poor degradative enzymatic systems, and others have described a direct role of earthworms in the decomposition of plant debris, and presume the existence of their own digestive enzymatic activities. Urbásek and Pizl (1991) described the presence of active amylase,

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0038-0717/99/$ - see front matter
PII: S0038-0717(99)00092-9

laminarinase, lichenase, cellulase, glucoamylase and xylanase in the gut of five earthworm species. Since in their experiments gut walls were washed using toluene, these enzymes are presumed to be of worm origin. Zhang et al. (1993) made a systematic study of the glucidic enzymes in a tropical earthworm *Pontoscolex corethrurus* concluding that it possesses a weak but quite complete enzyme system. In vitro cultures of foregut and midgut wall samples allowed the detection of laminarinase, amylase, maltase, N-acetylglucosaminidase, xylanase, carboxymethylcellulase and galactomannanase in this species. In contrast, they reported the absence of cellulase and mannanase activities indicating that this earthworm does rely on microflora to degrade cellulose and mannan. Similar studies were carried out by Lattaud et al. (1997a, b) in *Polypheretima elongata* and in *Millsonia anomala*, concluding that, in both species, the glucosidic activities were higher than those previously found in *P. corethurus*. In vitro tissue culture of gut wall demonstrated that *P. elongata* can synthesise by itself its extra and intracellular enzymes (Lattaud et al., 1997a), whereas *M. anomala*, as *P. corethurus*, cannot produce cellulase and mannanase and uses the digestive enzymatic capabilities of the ingested microflora instead (Lattaud et al., 1997b).

In this study the presence of xylanolytic enzymes in extracts from *Eisenia andrei*, and characterisation of these enzymes in relation to pH, temperature and kinetics parameters were investigated. In addition, the influence of the presence of xylan on the stimulation of the enzymatic activity, particularly in the case of xylanase, was also studied. These results provide important information for understanding the role of *E. andrei* in vermicomposting of lignocellulosic wastes.

2. Materials and methods

2.1. Preparation of samples

Empty gut adult earthworms (kept 6 h at filter paper) from our laboratory stock (fed on cow manure — ST, standard medium — and maintained at room temperature) were collected, weighed, washed in ice-cold phosphate 10 mM pH 7.0 buffer, and homogenised (1/5, w/v) under cooling in the same solution using a Potter–Elvejhem (2 min; 1500 rev min^{-1}). The homogenates were then centrifuged for 20 min at $12\,000 \times g$ at 4°C, and the supernatant fractions were used for biochemical assays.

To determine the effect of xylan on the xylanolytic activities, earthworms were fed for 30 d on paper-pulp mill sludge (PMS), a xylan-rich medium (Elvira et al., 1996). PMS was obtained from ENCE wastewater plant, a Kraft paper-pulp industry located in Pontevedra, Spain. The sludge had been amended with pig slurry according to Elvira et al. (1995).

Samples of fresh PMS and ST were taken, homogenised and centrifuged as those of earthworms for xylanase activity assays.

2.2. Enzyme assays and protein determination

Total enzymatic activity of xylanolytic enzymes was determined using the supernatant fractions from earthworms *E. andrei* fed on dung (standard medium) and on paper mill sludge amended with pig slurry.

Xylanase (E.C.3.2.1.8) activity was determined by the dinitrosalicylic acid method (Miller, 1959), using soluble birch wood xylan as substrate. One unit of enzyme (U) was defined as the amount of enzyme that releases one μmol of reducing sugar (expressed as xylose equivalents) min^{-1}. A typical assay for β-D-xylosidase (E.C.3.2.1.37) activity was performed in a reaction mixture containing 250 μl of 0.1 M citrate buffer, pH 3.0, 225 μl of 6.5 mM *p*-nitrophenyl-β-D-xylopiranoside as substrate and 25 μl of enzymatic sample. The same procedure was followed for the assay of α-L-arabinosidase (E.C.3.2.1.55) but using 0.1 M phosphate buffer, pH 6.5 and 5 mM *p*-nitrophenyl-α-L-arabinoside and for the assay of β-D-glucuronidase (E.C.3.2.1.31) using citrate buffer pH 4.0, and 2.0 mM *p*-nitrophenyl-glucuronide. Mixtures were incubated at 37°C during 30 min, reactions were stopped by addition of 500 μl of 0.2 M Na_2CO_3 and the released *p*-nitrophenyl detected at 400 nm.

Acetyl xylan esterase (E.C.3.1.1.6) was determined using 5 mM *p*-nitrophenyl-acetate dissolved in dimethyl sulfoxide as substrate, following the method described by Johnson et al. (1988), with some modifications. Initially, 100 μl of 0.5 M potassium phosphate buffer pH 6.5, 20 μl of enzyme solution and distilled water in a total volume of 900 μl were added to test tubes. To initiate the reaction, 100 μl of substrate were added, and after 10 min at 37°C the absorbance at 420 nm was determined. Since *p*-nitrophenyl-acetate may suffer nonenzymatic deesterification in alkaline pH (Lee et al., 1987), in experiments involving acetyl esterase activity, assays other than those in pH dependence studies were carried out at pH 6.5. One unit (U) of enzyme was defined as the amount of enzyme which releases 1 μmol *p*-nitrophenol min^{-1} under the assay conditions for each enzymatic activity. Concentration of *p*-nitrophenol was calculated in each case from standard curves obtained under assay conditions.

In every assay, both reagent blank and enzyme control readings were subtracted from test readings. Protein concentration was determined according to the method by Lowry et al. (1951) using bovine serum albumin as standard.

Table 1
Total activity (U g^{-1} tissue) of xylanolytic enzymes in earthworms fed on cow manure (ST) or on paper-pulp mill sludge[a] (PMS), a xylan-rich medium

	Earthworms fed on ST/U g^{-1} tissue (mean ± S.E.M.)[b]	Earthworms fed on PMS/U g^{-1} tissue (mean ± S.E.M.)[b]
Xylanase[c]	2.06 ± 0.154 (100%)	2.73 ± 0.135 (133%)
Acetyl xylan esterase[c]	0.55 ± 0.037 (100%)	0.97 ± 0.253 (176%)
β-D-glucuronidase[c]	0.17 ± 0.008 (100%)	0.32 ± 0.023 (188%)
α-L-arabinosidase[c]	0.04 ± 0.003 (100%)	0.06 ± 0.001 (150%)
β-D-xylosidase[c]	0.03 ± 0.005 (100%)	0.07 ± 0.005 (233%)

[a] Earthworms were fed on paper-pulp mill sludge amended with pig slurry for 35 d.
[b] Standard error of means. The data are averages of three experiments.
[c] All values in the same row are significantly different ($P < 0.01$). HSD Tukey's test.

2.3. Partial characterisation of xylanolytic enzymes

Biochemical characterisation was performed in extracts of earthworms that were fed on cow manure (standard medium), unless otherwise specified. The pH dependence of the enzymes was determined by replacing the buffer in the standard assay mixtures by citrate or phosphate buffers at pH 3.0–5.0 and 5.0–8.0, respectively. After 60 min reaction at 37°C the activity was measured as described above. Assays to determine the optimal temperature for each enzyme were carried out as described before, except that the reaction was performed at temperatures ranging from 4 to 60°C. In these experiments blanks (without enzymatic sample) were maintained under the same conditions as test tubes and used as controls of nonenzymatic hydrolysis.

Kinetic studies were performed under standard assay conditions but varying the concentration of the specific substrates. Xylanase was assayed using soluble birch wood xylan at concentrations ranging from 1.6 to 32 mg ml^{-1}. Substrate concentrations for β-D-xylosidase and β-D-glucuronidase ranged from 0.18 to 4 mM. In the case of α-L-arabinosidase and acetyl xylan esterase, substrate concentration ranged from 0.34 to 8 mM and 0.1 to 1 mM, respectively. Apparent Michaelis–Menten constant (K_m) and maximal velocity (V_{max}) values were calculated using the nonlinear regression data analysis programme 'Ultrafit' (Biosoft, Cambridge, UK). Kinetic parameters for xylanase activity were also determined in extracts from earthworms fed on paper mill sludge amended with pig slurry.

All data are the means of three independent experiments. Statistical procedures were carried out with the software packages SPSS for Windows. One-way ANOVA and HSD Tukey's test was employed to identify significant differences in the total activity of xylanolytic enzymes in earthworms fed on standard medium or fed on paper-pulp mill sludge.

3. Results

3.1. Activity of xylanolytic enzymes in E. andrei

The values of total enzymatic activity, measured as U g^{-1} of tissue, are shown in Table 1. Xylanase displayed the highest total activity. Total acetyl esterase activity was found to be 0.55 U g^{-1}. β-glucuronidase,

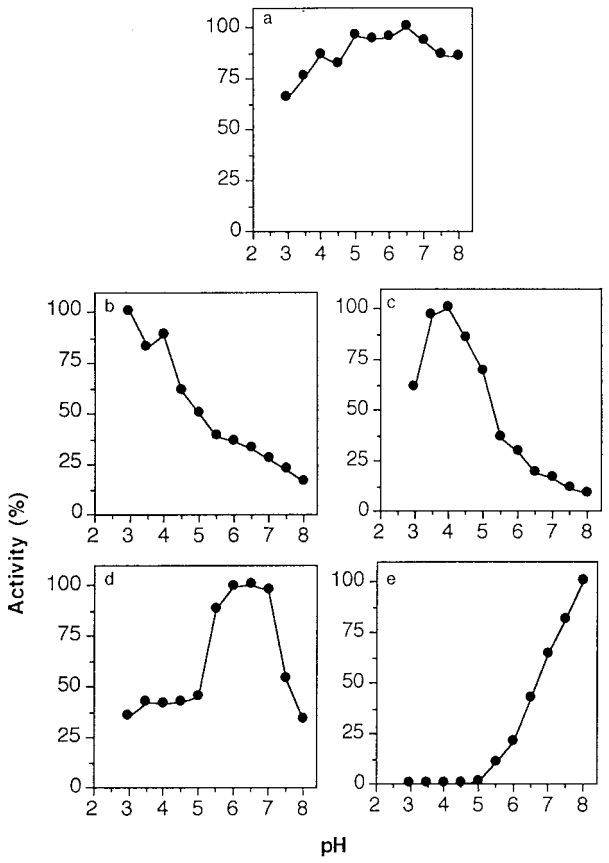

Fig. 1. Effect of pH on xylanolytic enzymes. (a) xylanase; (b) β-D-xylosidase; (c) β-D-glucuronidase; (d) α-L-arabinosidase; (e) acetyl xylan esterase. The data are averages of three independent experiments.

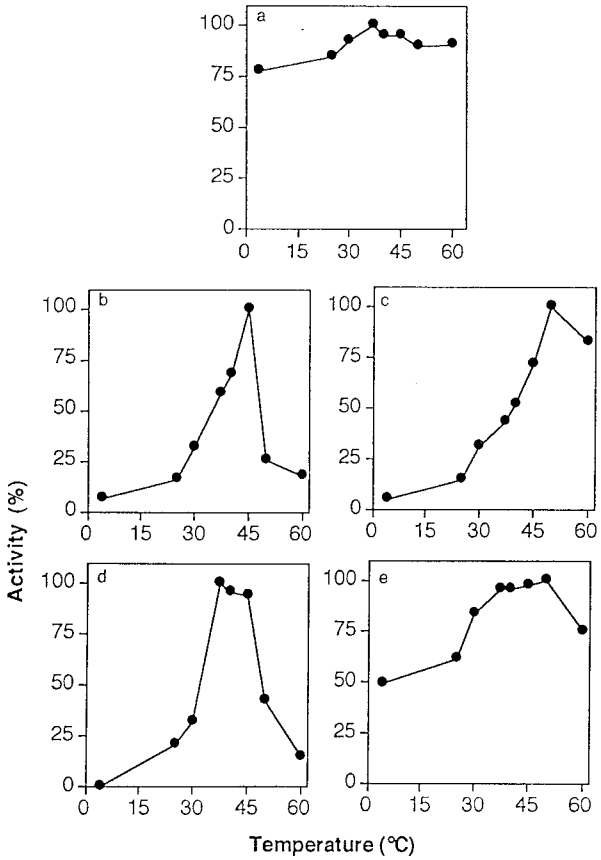

Fig. 2. Effect of temperature on xylanolytic enzymes. (a) xylanase; (b) β-D-xylosidase; (c) β-D-glucuronidase; (d) α-L-arabinosidase; (e) acetyl xylan esterase. The data are averages of three independent experiments.

α-arabinosidase and β-xylosidase showed lower activities (ranged from 0.03 to 0.17 U g^{-1}). In the presence of xylan containing medium, a statistically significant increase ($P < 0.01$) was observed: In the case of xylanase activity, the increase was 33%, while α-L-arabinosidase activity was enhanced by 50%. An increase of more than 75% was observed for β-D-xylosidase, β-D-glucuronidase and acetyl xylan esterase.

Total xylanase activity (U g^{-1} dry weight) was 10-fold greater in extracts of earthworms fed on PMS than in the PMS, and 180-fold in earthworms fed on cow manure than in this standard medium.

3.2. Effect of pH on xylanolytic enzymes

The effect of pH on xylanolytic enzymes from earthworms fed on standard medium is shown in Fig. 1. Xylanase showed a wide pH activity curve with a maximum at 6.5 and activities higher than 70% at pH values between 4.0 and 8.0 (Fig. 1a). β-D-xylosidase and β-D-glucuronidase had acidic optimum pH of 3.0 and 4.0, respectively (Fig. 1b,c). In contrast, α-L-arabinosidase showed a maximum in a neutral range of pH between 6.0 and 7.0 (Fig. 1d) and acetyl xylan esterase displayed the highest activity at pH 8.0 (Fig. 1e). The extracts of earthworms fed on PMS and ST showed the same ($P > 0.05$) optimum pH for each enzyme.

3.3. Effect of temperature on xylanolytic enzymes

All the enzymes described here displayed a high activity in the range 35–40°C. Xylanase showed its highest activity at 37°C but remained active over a wide range of temperatures from 4 to 60°C (Fig. 2a). In contrast, glycosidase enzymes showed narrower ranges of activity: β-xylosidase showed optimal temperature at 45°C (Fig. 2b); β-D-glucuronidase at 50°C (Fig. 2c), whereas α-L-arabinosidase showed the highest activity between 37 and 45°C (Fig. 2d). Acetyl xylan esterase

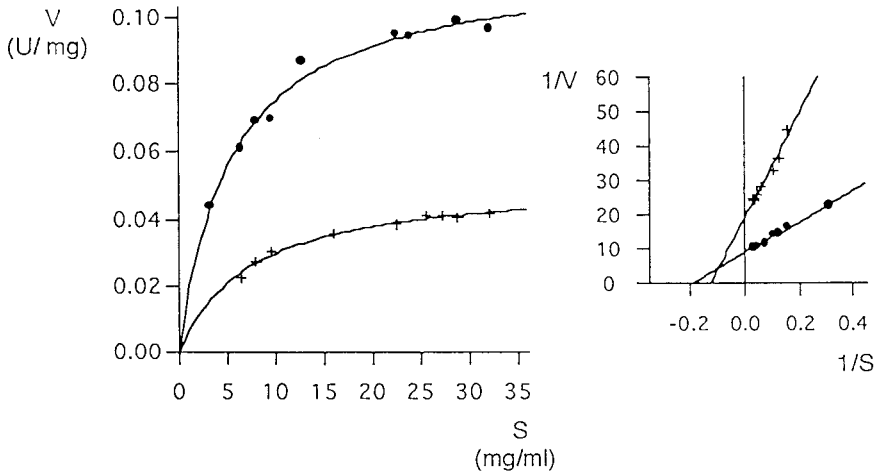

Fig. 3. Michaelis–Menten curves and Lineawever–Burk plots of xylanase activity using soluble xylan as substrate. (•) extracts from earthworms fed on cow manure; (+) extracts from earthworms fed on paper-pulp mill sludge. The data are averages of three independent experiments.

showed a temperature curve with a maximum at 50°C and retained more than 75% of the activity at temperatures between 30 and 60°C (Fig. 2e).

3.4. Kinetic studies

In the extracts of earthworms fed on standard medium, the apparent K_m values calculated from Lineweaver–Burk plots and expressed as mean ± S.E.M. ($n = 3$), were 1.24 ± 0.150 mM for β-D-xylosidase, 0.49 ± 0.117 mM for β-D-glucuronidase, 0.84 ± 0.174 mM for α-L-arabinosidase and 0.36 ± 0.092 mM for acetyl xylan esterase. V_{max} values were 0.003 ± 0.0001 U mg^{-1} in the case of β-D-xylosidase and α-L-arabinosidase, 0.013 ± 0.0010 U mg^{-1} for β-D-glucuronidase and 0.190 ± 0.0190 U mg^{-1} for acetyl esterase.

Kinetic studies of xylanase activity were performed on extracts from earthworms fed either on dung or on paper-pulp mill sludge amended with pig slurry. Michaelis–Menten curves and Lineweaver–Burk plots are represented in Fig. 3. The apparent K_m values were 6.91 ± 1.120 mg ml^{-1} in the case of the control earthworms, and 5.60 ± 1.070 mg ml^{-1} in the case of the earthworms fed on paper mill sludge. The estimated V_{max} in control extracts was 0.05 ± 0.002 U mg^{-1}, whereas in the case of extracts from earthworms fed on paper-pulp medium it was much higher (0.12 ± 0.006 U mg^{-1}; $P < 0.01$).

4. Discussion

A considerable amount of xylan is released from wood during pulp processing, and this waste may cause ecological problems (Vyas et al., 1990). On an industrial scale, the pulp mill sludge is usually processed by destructive methods such as incineration and land filling practices, involving the loss of a profitable resource (Tirsch, 1990). An alternative management practice could be the transformation of the sludge by vermicomposting (Elvira et al., 1995, 1996b, 1997).

We have demonstrated the presence of the xylanolytic enzymes xylanase, β-D-xylosidase, β-D-glucuronidase, α-L-arabinosidase and acetyl xylan esterase in extracts from the earthworm *E. andrei*. As others (Urbásek and Chalupsky, 1991; Sustr and Chalupsky, 1996), we assume that the enzyme activity measured in a homogenate of whole earthworm body is a measure of digestive activity in vivo. Thus, despite their origin (e.g. gut wall cells, digestive glands as well as gut-wall-associated microflora), the occurrence of active xylanolytic enzymes in extracts of *E. andrei* indicates that xylan is degraded in their gut and earthworm activity contributes to breakdown hemicellulose.

The determination of total enzymatic activity, measured as U g^{-1} of tissue, showed that the enzyme xylanase was the most active, followed by acetyl xylan esterase and β-D-glucuronidase, whereas α-L-arabinosidase and β-xylosidase displayed lower total activity. In all of the cases, an increase of the total enzymatic activity was detected when xylan was present in the culture medium. The increase in the enzyme activities could be simply attributed to the proliferation of gut-associated microorganisms, or possibly to the induction of the synthesis or the secretion of the enzymes as described by Biely (1985) for xylanolyitc enzymes in noncellulolytic bacteria and yeasts. Recently, De Groot et al. (1998) observed that endo-1,4-β-xylanase from a fungus species (*Agaricus bisporus*) was expressed at a high activity when colonies were transferred from glucose to xylan containing medium. However, transfer from glucose to compost yielded a much stronger and constant induction, suggesting that the gene is induced by compost-specific factors rather than by the substrates they act upon.

Our studies, using soluble xylan as substrate, indicated that xylanase showed maximal activity at pH 6.5 and retained 90–95% of the activity in the pH range 5.0–7.0. Urbásek and Pizl (1991), studying xylanase activity in extracts from five different earthworm species, reported the optimal pH to be 7.0–7.5. Xylan esterase and α-L-arabinosidase also showed optimal activity at pH 6.0–7.0. In contrast, β-xylosidase and β-glucuronidase displayed activity only at acidic pH values.

The xylanolytic activities in extracts of earthworms fed on standard medium (without xylan) displayed typical Michaelis–Menten curves. Previous studies of xylanase showed a high disparity of K_m and V_{max} values; probably due to the different origin of the substrates used in the in vitro assays. Using birch wood xylan the K_m value in *E. andrei* (6.91 ± 1.120 mg ml^{-1}) is comparable to that described in *Penicillium chrysogenum* using the same substrate (4.2 mg ml^{-1}) (Haas et al., 1992). According to our data, in homogenates of earthworm fed on standard medium xylanase displayed a V_{max} of 0.05 U mg^{-1}. Deleporte and Charrier (1996) reported that larvae of two forest sciarid species, *Bradysia confinis* and *Plastociara falcifera*, were able to degrade xylan, showing in homogenates a specific activity of 0.067 and 0.3 U mg^{-1}, respectively.

There is very little reported information about the kinetic parameters of the α-L-arabinosidase and β-glucuronidase xylanolytic enzymes. Dekker (1983) reported a K_m of 1.02 mM for *Trichoderma reesei* β-xylosidase, slightly lower than that obtained here for *E. andrei* with the same substrate (1.24 mM).

The kinetic parameters for xylanase were also determined in extracts of earthworms fed in xylan containing medium, since β-xylanase is considered the key enzyme of the complex that initiates the hydrolysis of xylan to oligosaccharides (Haas et al., 1992). In the

presence of xylan, an increase in the substrate affinity (and thus a lower K_m) as well as a higher rate of substrate hydrolysis (higher V_{max}) were observed. The latter could be explained as a higher concentration of enzymatic protein present in earthworms fed on xylan.

The occurrence of active xylanolytic enzymes in extracts from *E. andrei* together with the observation that these activities are higher when earthworms were fed on paper mill sludge, give us confidence on the utility of vermicomposting in the management of wastes from the paper industry and point out *E. andrei* as an interesting promotor in the degradation of hemicellulose.

Acknowledgements

We thank V.S. Martínez-Zorzano and M. Pérez-Losada for the critical review of this manuscript and Ms. Jacqueline Kenny for English supervision. This work was supported in part by a grant from Ministerio de Educación y Ciencia, Spain. A. M. and L.S. were supported by pre-doctoral fellowships from the Spanish Ministerio de Educación y Ciencia.

References

Bachmann, S.L., McCarthy, A.J., 1991. Purification and cooperative activity of enzymes constituting the xylan-degrading system of *Thermomonospora fusca*. Applied Environmental Microbiology 57, 2121–2130.

Biely, P., 1985. Microbial xylanolytic systems. Trends in Biotechnology 3, 286–290.

De Groot, P.W.J., Basten, D.E.J.W., Sonnenberg, A.S.M., Van Griensven, L.J.L.D., Visser, J., Schaap, P.J., 1998. An endo-1,4-β-xylanase-encoding gene from *Agaricus bisporus* is regulated by compost-specific factors. Journal of Molecular Biology 277, 273–284.

Dekker, R.F.H., 1983. Bioconversion of hemicellulose: aspects of hemicellulase production by *Trichoderma reesei* QM 9414 and enzymic saccharification of hemicellulose. Biotechnology and Bioengineering 25, 1127–1146.

Deleporte, S., Charrier, M., 1996. Comparison of digestive carbohydrases between two forest sciarid (Diptera: Sciaridae) larvae in relation to their ecology. Pedobiologia 40, 193–200.

Elvira, C., Goicoechea, M., Sampedro, L., Mato, S., Nogales, R., 1996. Bioconversion of solid paper-pulp mill sludge by earthworms. Bioresource Technology 57, 173–177.

Elvira, C., Sampedro, L., Domínguez, J., Mato, S., 1997. Vermicomposting of wastewater sludge from paper pulp industry with nitrogen rich materials. Soil Biology & Biochemistry 29, 759–762.

Elvira, C., Domínguez, J., Sampedro, L., Mato, S., 1995. Vermicomposting for the paper pulp industry. BioCycle, June, 60–63.

Haas, H., Herfurth, E., Stöffler, G., Redl, B., 1992. Purification, characterisation and partial amino acid sequences of xylanase produced by *Penicillium chrysogenum*. Biochimica et Biophysica Acta 1117, 279–286.

Johnson, K.G., Fontana, J.D., MacKenzie, C.R., 1988. Measurement of acetylxylan esterase in *Streptomyces*. Methods in Enzymology 160, 551–560.

Lattaud, C., Locati, S., Mora, P., Rouland, C., 1997a. Origin and activities of glycolytic enzymes in the gut of the tropical geophagous earthworm *Millsonia anomala* from Lamto (Cote d'Ivoire). Pedobiologia 41, 242–251.

Lattaud, C., Zhang, B.G., Locati, S., Rouland, C., Lavelle, P., 1997b. Activities of the digestive enzymes in the gut and in tissue culture of a tropical geophagous earthworm *Polypheretima elongata* (Megascolecidae). Soil Biology & Biochemistry 29, 335–339.

Lee, H., To, R.J.B., Latta, R.K., Biely, P., Schneider, H., 1987. Some properties of extracellular acetylxylan esterase produced by the yeast *Rhodotorula mucilaginosa*. Applied and Environmental Microbiology 53, 2831–2834.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. Journal of Biological Chemistry 193, 265–275.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Analytical Chemistry 31, 426–428.

Puls, J., Borchmann, A., Gottschalk, D., Wiegel, J., 1988. Xylobiose and xylooligomers. Methods in Enzymology 160, 528–536.

Sustr, V., Chalupsky, J., 1996. Activity of digestive enzymes in two species of potworms (Oligochaeta, Enchytraeidae). Pedobiologia 40, 255–259.

Tirsch, F.S., 1990. Pulp and paper effluent management. Research Journal of Water Pollution Control Foundation 62, 478–484.

Urbásek, F., Chalupsky Jr., J., 1991. Activity of digestive enzymes in 4 species of Enchytreidae (Oligochaeta). Revue d'Ecologie et de Biologie du Sol 28, 145–154.

Urbásek, F., Pizl, V., 1991. Activity of digestive enzymes in the gut of five earthworm species (Oligochaeta; Lumbricidae). Revue d'Ecologie et de Biologie du Sol 28, 461–468.

Vyas, P., Chauthaiwale, V., Phadatare, S., Deshpande, V., Srinivasan, M.C., 1990. Studies on the alkalophilic *Streptomyces* with extracellular xylanolytic activity. Biotechnology Letters 12, 225–228.

Zhang, B.G., Rouland, C., Lattaud, C., Lavelle, P., 1993. Activity and origin of digestive enzymes in gut of tropical earthworm *Pontoscolex corethrurus*. European Journal of Soil Biology 29, 7–11.