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Use of Corncob for Endoxylanase Production by Thermophilic Fungus *Thermomyces lanuginosus* IOC-4145

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Abstract

The production of cellulase-free endoxylanase by the thermophilic fungus *Thermomyces lanuginosus* was investigated in semisolid fermentation and liquid fermentation. Different process variables were investigated in semisolid fermentation, employing corncob as the carbon source. The best results were with the following conditions: grain size = 4.5 mm, solid:liquid ratio = 1:2, and inoculum size = 20% (v/v). Corncob, xylan, and xylose were the best inducers for endoxylanase production. Additionally, organic nitrogen sources were necessary for the production of high endoxylanase activities. The crude enzyme had optimum activity at pH 6.0 and 75°C, displaying a high thermostability. The apparent K_m and V_{max} were 1.77 mg of xylan/mL and 21.5 U/mg of protein, respectively.

Index Entries: Xylanases; *Thermomyces lanuginosus*; corncob; liquid fermentation; semisolid fermentation.

Introduction

Xylanases have a wide range of potential biotechnological applications. They can be used in bioconversion of xylan-containing lignocellulosic materials to D-xylose, which can be converted to a variety of bioproducts with high aggregate value. The xylanases have also been used in the production of dissolving pulp *(1)* and in the pretreatment of pulp for enhancing brightness *(2,3)*. For this purpose, the enzymes should be free from cellulase activity, which would destroy the pulp quality; they must be stable and active at high temperatures and at alkaline pH; and high enzyme yields at a very low cost should be easily produced *(4)*.

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The technique of semisolid fermentation involves the growth and metabolism of microorganisms on moist solids in the absence, or near absence, of any free-flowing water. This technique offers distinct advantages over liquid fermentation including economy of the space needed for fermentation, simplicity of the fermentation media, higher product yields, aeration facilitated by spaces between substrate particles, lesser volume of solvent needed for product recovery, and easier control of contamination owing to the low moisture level *(5–7)*.

Fungal systems are being investigated for the production of enzymes by semisolid fermentation. This mode of fermentation, which is closer to the natural habitats of microbes, may prove more efficient in the production of bioproducts *(6,8)*. In general, the carbon source has been estimated as the major cost factor in enzyme production. Because pure xylan is too expensive for use in industrial-scale fermentation, one possibility for reduction of production costs is to use inexpensive waste materials *(9)*. Potential hemicellulosic sources include agricultural and forest wastes such as corncob, sugar cane bagasse, wheat bran, rice straw, leaves, and stalks.

The use of thermostable xylanases for enzymatic hydrolysis or pretreatment of pulp at high temperatures might help in overcoming technical and economic feasibility. Moreover, as the thermophilic fungus grows at high temperatures, there is reduced risk of microbial contamination and cooling requirements for the fermentation processes are not needed *(10)*.

Xylanase production has been detected by mesophilic fungi, e.g., *Aspergillus niger (8,11,12)*, *Aspergillus phoenicus (12)*, *Penicillium canescens* 10-10c *(13)*, as well as by thermophilic fungi, e.g., *Thermoascus aurantiacus (14–16)*, and *Thermomyces lanuginosus (14,17)*. Recently, *T. lanuginosus* has been described as an excellent enzyme producer, and several characteristics of the produced enzyme under liquid fermentation have been described *(10,17,18)*.

In this context, we have studied the effect of different factors, such as incubation period, inoculum size, and carbon source, on the production of an extracellular thermostable endoxylanase in semisolid fermentation and liquid fermentation by *T. lanuginosus* IOC-4145. The optimum activity and stability, in terms of pH and temperature of the enzyme produced, were studied aiming at the partial characterization of this enzyme.

Materials and Methods

Materials

All chemicals were of analytical grade and obtained from Sigma (St. Louis, MO) unless otherwise stated. Agar-agar, meat extract, maltose, ammonium chloride, and ammonium sulfide were purchased from Merck.

Organism and Growth Conditions

The fungal strain used was isolated from soil at the IBILCE/UNESP, and identified by FIOCRUZ (Fundação Instituto Oswaldo Cruz) under the

code IOC-4145. This strain was maintained on slants of oats agar (50.0 g/L oats, 30.0 g/L agar) at 4°C and on slants containing sand at 4°C. Spore suspensions were prepared by adding 3 mL of 0.1% Tween-80 to slant cultures and the surface was gently rubbed with a sterilized wire loop. The composition of the production medium was as follows: 10.0 g/L of peptone, 10.0 g/L of meat extract, 10.0 g/L of NaCl, and 1.0 g/L of KH_2PO_4.

Inoculum Size

Corncob (15 g) moistened with 30 mL of medium in 500-mL Erlenmeyer flasks were autoclaved and inoculated with 10, 20, and 30% (v/v) of mycelium suspensions based on liquid medium after 20 h of cultivation. The fungus was grown in a medium containing glucose as carbon source. The enzyme was extracted and assayed for each set, following incubation at 45°C.

Xylanase Production in Liquid Fermentation

(10% [v/v]) Mycelium suspension or 10^6 spores/mL was used to inoculate conical flasks (500 mL) containing 3 g of corncob or 1 g of birchwood xylan, or other carbon sources in 100 mL of production medium. After inoculation, the flasks were incubated at 45°C on a rotary shaker at 150 rpm.

Xylanase Production in Semisolid Fermentation

(20% [v/v]) Mycelium suspension or 10^6 spores/mL was used to inoculate conical flasks (500 mL) containing 15 g of corncob and 30 mL of production medium. After cultivation at 45°C, the enzyme was extracted through the addition of 100 mL of distilled water and stirred on a rotary shaker at 150 rpm at room temperature. The culture medium of liquid fermentation and the crude extract of semisolid fermentation was vacuum filtered using filter paper.

Analytical Methods

During the cultivation, two or more flasks were sampled daily and analytical determinations were assayed in triplicate.

Enzyme Assays

Endoxylanase was assayed using birchwood xylan as substrate. One milliliter of the reaction mixture contained 0.1 mL of appropriately diluted enzyme solution and 0.9 mL *(19)* of a 0.5 or 1% suspension of xylan in an appropriate buffer, and incubated at 60–75°C for 3–10 min as stated in the figures. The reducing sugars were assayed by the dinitrosalicylic acid procedure *(20)*, with xylose as standard. The carboxymethylcellulolytic activity was assayed by a similar method, utilizing 0.5% CMCellulose in acetate buffer (0.1 *M*, pH 5.0) and incubated at 60°C for 10 min; glucose was used as standard. The liberated xylose or glucose was measured spectropho-

tometrically at 540 nm. β-Xylosidase activity was assayed *(21)* using *p*-nitrophenol as standard. One unit of enzyme activity was defined as 1 μmol of reducing sugars/*p*-nitrophenol released per minute under the described assay conditions. Protein concentration was measured by Lowry *(22)* using bovine serum albumin as standard.

Biomass

Dry weight in liquid cultures was measured by filtering the mycelium grown on birchwood xylan 1% (w/v) as previously described and dried at 50°C.

Kinetic Determinations

The initial rate of xylan hydrolysis using crude enzymes was determined with birchwood xylan at various concentrations (0.19–1.08%). A kinetic microcomputer program *(23)* and the Lineweaver-Burk method estimated the apparent kinetic constants K_m and V_{max}.

Physicochemical Parameters

The optimum pH of endoxylanase activity was measured using substrate prepared with the Universal buffer adjusted to an initial pH of 3.0–11.0. After incubation, the reducing sugars were assayed as described previously. Endoxylanase activity was measured over an ample temperature range (35–90°C), performing the standard reaction for determining optimum temperature. The effect of pH on the stability of the xylanase was studied by incubating the enzyme without substrate, with buffers (pH range of 3.0–10.0) for different times. After incubation, the remaining endoxylanase activity was determined. The effect of temperature on the enzyme activity was determined by incubation of crude extract at 50–90°C for different times, and then the residual activity was measured.

Results and Discussion

Kinetics of Xylanase Production in Liquid Fermentation

The kinetic profile of enzymatic production of *T. lanuginosus* IOC-4145 in liquid medium was obtained after cultivation under agitation, at 45°C for 168 h. The maximum peak of endoxylanase production occurred at 96 h (Fig. 1) of cultivation in birchwood xylan (about 10 U/mL) or corncob (about 17 U/mL), independent of the grain size used (4.5 or 6.0 mm). Although it would be expected to observe a higher enzyme production in a medium containing birchwood xylan, since it is a more promptly utilized substrate for induction/production of xylanases, the best results of enzyme activity were obtained in the medium containing corncob as feedstock. It is possible that the producing strain is able to use other corncob components beside xylan for growth and induction of its enzymes. Similar results were obtained *(10,24)* using several lignocellulosic materials. It is worth noting

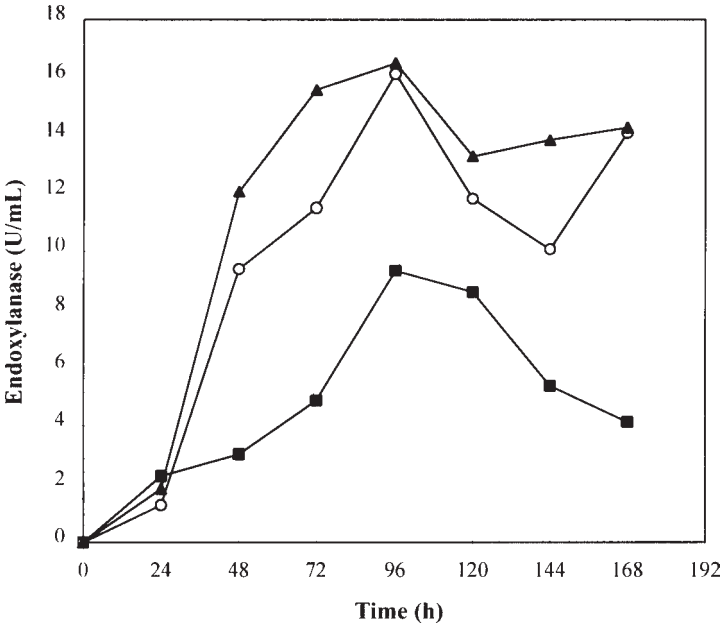

Fig. 1. Production of endoxylanase as a function of time during growth of *T. lanuginosus* IOC-4145 in liquid fermentation on xylan (—■—), corncob ϕ = 4.5 mm (—○—), and corncob ϕ = 6.0 mm (—▲—). The enzymatic activity was assayed in 0.5% xylan in acetate buffer 0.1 *M* at 60°C for 10 min.

that no cellulase activity from either corncobs or xylan could be detected in any of the crude extract. The lower levels of endoxylanase production in birchwood xylan-containing medium, when compared with those obtained in corncob medium, may be related to the source of xylan used as carbon source *(25)*.

Figure 2 shows the growth profile and endoxylanase and β-xylosidase production in liquid fermentation, using 1% xylan as substrate. It can be observed that both enzymes display different kinetic profiles. Whereas β-xylosidase peaked (0.152 U/mL) at 48 h of cultivation, endoxylanase presented maximum activity during the stationary phase (10 U/mL) at 96 h. This unexpected phenomenon, i.e., β-xylosidase exhibiting activity before endoxylanase, could be ascribed to the necessity of inducers for the endoxylanase production, which would be liberated with the action of β-xylosidase in the first hours of the fermentation.

Kinetics of Xylanase Production in Semisolid Fermentation

The kinetic profile of enzymatic production of *T. lanuginosus* IOC-4145 in semisolid medium was obtained after cultivation at 45°C for 144 h. The maximum of endoxylanase production was reached at different times depending on the grain size and also on the solid:liquid ratio. The maximum endoxylanase production (120 U/mL) was obtained in the medium containing corncobs (ϕ = 4.5 mm), with a solid:liquid ratio of 1:2, after 72 h of cultivation (Fig. 3). On the other hand, the fermentation carried out using

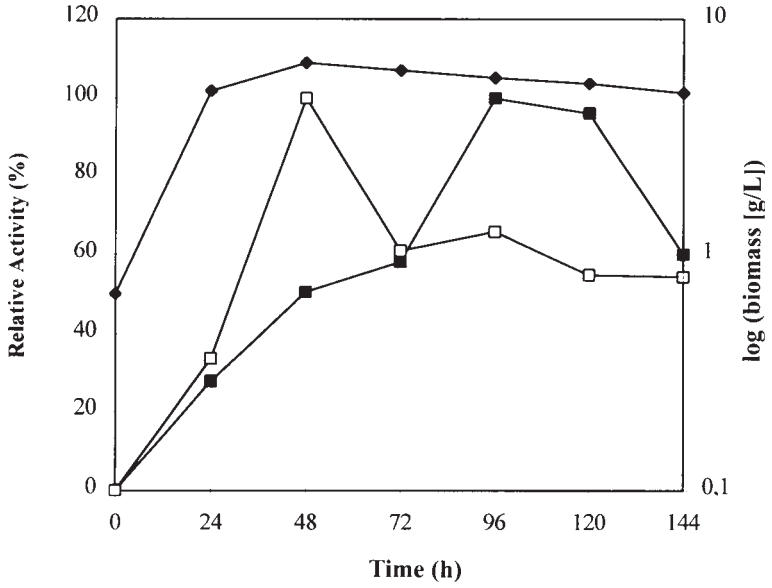

Fig. 2. Growth profile (—◆—), endoxylanase (—■—), and β-xylosidase (—□—) production by *T. lanuginosus* IOC-4145 in liquid medium containing 1% xylan. The highest value of enzyme production corresponding at 100%. The enzymatic activity was assayed in 0.5% xylan in acetate buffer 0.1 *M* at 60°C for 10 min.

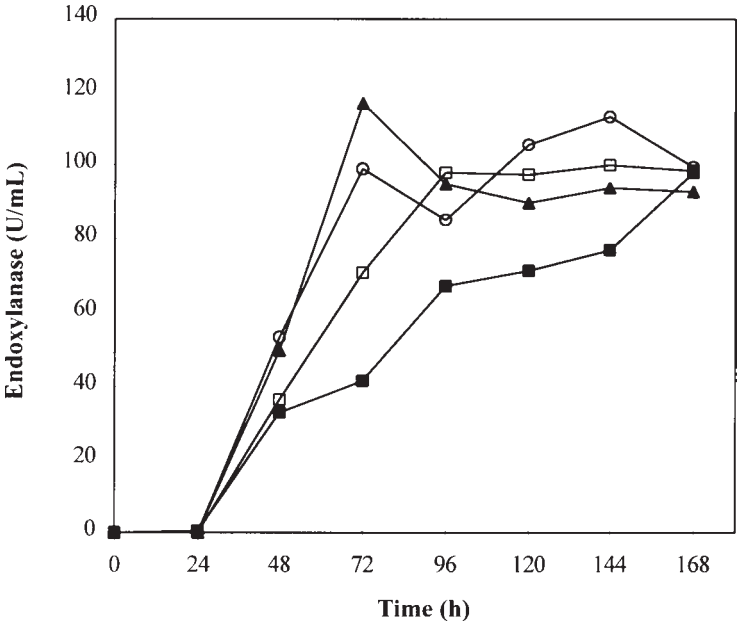

Fig. 3. Production of endoxylanase as a function of time during growth of *T. lanuginosus* IOC-4145 in semisolid fermentation using corncob at different solid:liquid ratios. (—□—), ϕ = 6.0 mm/1:1.5; (—○—), ϕ = 4.5 mm/1:1.5; (—■—), ϕ = 6.0 mm/1:2; (—▲—), ϕ = 4.5 mm/1:2. The enzymatic activity was assayed in 0.5% xylan in acetate buffer 0.1 *M* at 60°C for 10 min.

a grain size of 6.0 mm showed a delay in the enzyme production, being more accentuated as the water content increased. Neither the β-xylosidase nor the cellulase could be detected in the crude extract.

When compared with other reports employing different lignocellulosic materials *(8,11)*, *T. lanuginosus* IOC-4145 was shown to be a promising fungus for endoxylanase production. The production of xylanases by five *Aspergillus* soil isolates on semisolid cultures, supplemented with corncob, revealed a xylanase level between 35 and 54 U/mL *(8)*. The performance reached by *Chaetomium globosum* in semisolid fermentation led to an enzyme level of about 20 U/mL, after 48 h of cultivation *(11)*. The enzyme levels obtained by *T. lanuginosus* are almost four times higher than those related to other fungi in semisolid fermentation.

Liquid media did not increase enzyme production. On the other hand, semisolid fermentation improved both enzyme production and enzyme excretion. The influence of culture medium composition on biosynthesis and excretion of enzymes has already been reported. It could also be considered that the diffusion of catabolites and enzymatic inhibitors on semisolid medium is more difficult owing to its physical properties *(26)*. This behavior could partially explain the high activity levels obtained in semisolid fermentation by *T. lanuginosus* IOC-4145.

The differences in enzyme yields, using different lignocellulosic materials, could be owing to the nature of substrates and their pretreatments, presence of activators or inhibitors, surface area, pore size, different origin, mode of fermentation, and optimum cultivation conditions, which influence profoundly the extracellular enzyme production *(16,17)*.

Effect of Preinoculum Size and Successive Extractions in Semisolid Fermentation

Three ratios of the preinoculum used showed good performance (Fig. 4), but the ratio of 20% (v/v) displayed the maximum value (about 2400 U/[L·h]) for volumetric productivity. The inoculation with mycelium also allowed acceleration of the enzyme production when compared with those inoculated with spores (Figs. 3 and 4).

The use of sequential extractions demonstrated that part of the enzyme remained in the lignocellulosic material. The second extraction recovered about 40–50% of the initial extracted activity, whereas in the third almost 30% was recovered (Fig. 4). This is an important result, because real values of enzyme activity could be underestimated.

Determination of Apparent Kinetic Parameters K_m *and* V_{max}

The determination of K_m and V_{max} values revealed that the endoxylanase follows the standard Michaelis-Menten kinetics (data not shown). The values of apparent K_m and V_{max} are shown in Table 1. There were no significant differences between the values obtained either by Lineweaver-Burk or by kinetic *(23)* methods. In both cases, mathematical methods were

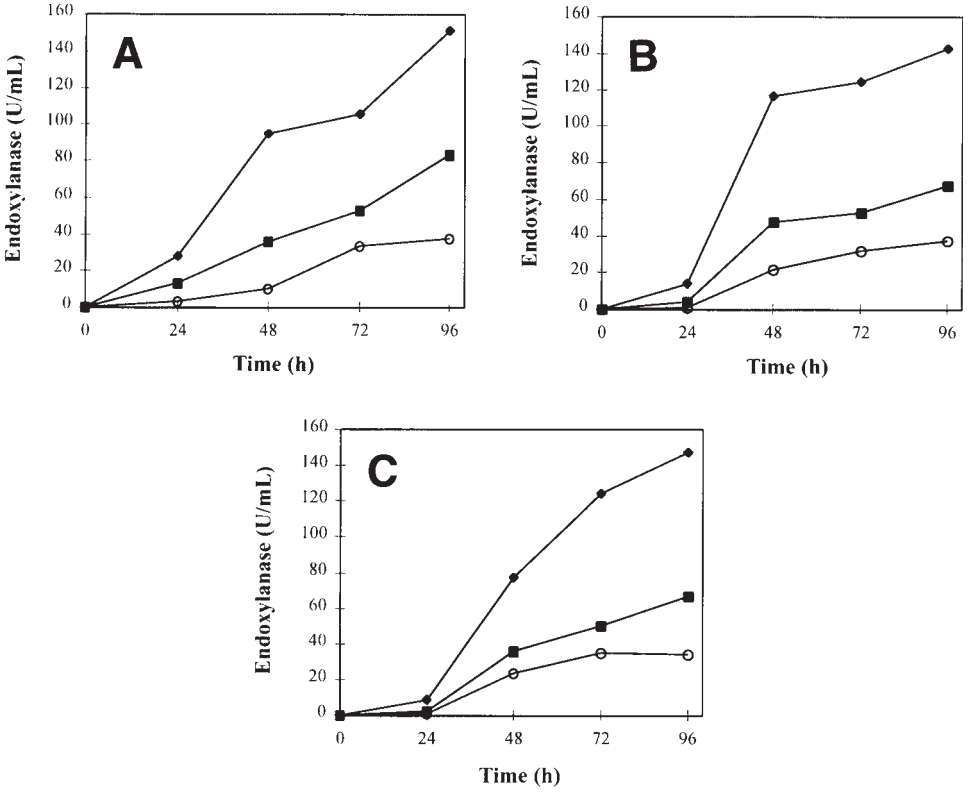

Fig. 4. Effect of the preinoculum size and successive extractions for the endoxylanase production by *T. lanuginosus* IOC-4145 in semisolid medium containing corncob (ϕ = 4.5 mm) at the ratio 1:2 for 96 h of cultivation. **(A)** 10%; **(B)** 20%; **(C)** 30% (v/v). Extractions: (—◆—) first; (—■—) second; (—○—) third. The enzymatic activity was assayed in 0.5% xylan in acetate buffer 0.1 *M* at 60°C for 10 min.

Table 1
Estimation Values of Apparent Kinetic Parameters

Parameter	Lineweaver-Burk	Kinetic	Relative error of equation parameters
K_m (mg xylan/mL)	1.78	1.76	0.26%[a]/5.3%[b]
V_{max} (U/mg)	21.50	21.45	0.26%[a]/1.4%[b]

[a]Error of Lineweaver-Burk method.
[b]Error of kinetic microcomputer program.

used to estimate K_m and V_{max} values. Indeed, the calculated errors obtained by the kinetic software can be considered an effective error, because it considers the Michaelis-Menten equation (hyperbolic function) for the error estimation and not its inverse, as in other methods. This error was very low (5.3%) owing to the good experimental data obtained, which fitted almost exactly the hyperbolic function. The software used allowed us to verify a

good accordance between experimental and predicted values, thus minimizing the errors on K_m and V_{max}.

On the other hand, the error observed by the Lineweaver-Burk method was caused by considering the equation obtained through a mathematical tool, the inverse method (1/S and 1/V). In that case, it led to a minor error, because it did not translate the real biochemical phenomenon. That mathematical tool is quite useful as a guideline to young researchers, when the objective of their studies deals with difficult kinetic models. In our case, the results obtained with the Lineweaver-Burk method were almost identical to the values obtained by the previous method, because ours is a simple kinetic model, disregarding inhibition, competition, or multiple kinetic effects. That kind of kinetic behavior is observed for the xylanase of *T. lanuginosus*. The obtained data were so good that the error turned out to be almost insignificant.

The mean value of the apparent K_m estimated for endoxylanase (1.77 mg of xylan/mL) demonstrates that the enzyme produced has a high affinity by the substrate. After estimation of the kinetic parameters, the optimization of the enzyme assay led to the use of the xylan at 1% concentration, and 3 min for enzymatic reaction.

Influence of Carbon and Nitrogen Sources

The effect of different carbon sources in the endoxylanase production was also evaluated. Substrates with different structural composition were used, from simple sugars to polymeric materials. Maltose, sugarcane bagasse, carboxymethylcellulose, and xylitol poorly induced the endoxylanase production, reflected in a low specific activity (Fig. 5 and Table 2). Higher values of endoxylanase activity were obtained using corncob (266 ± 54 U/mL), and especially xylan (716 ± 20 U/mL) and xylose (537 ± 2 U/mL), as well as the specific activity, as illustrated in Fig. 5 and Table 2.

T. lanuginosus IOC-4145 could produce endoxylanase values in xylan or xylose-containing media, about 5–60 times higher than those produced by other fungi *(13,16,25,27,28)*, which is surely owing to the capacity of the microorganism to consume xylan and its main building monomer.

The influence of the nitrogen source at the C:N ratio of 40:1 was studied, utilizing organic and inorganic sources for the enzyme production. The inorganic sources (sodium nitrate, ammonium sulfate, and ammonium chlorate) induced low amounts of endoxylanase, whereas organic sources led to a higher enzyme expression (Fig. 6). These results might indicate differences in the regulatory mechanisms of endoxylanase production.

Physicochemical Parameters

The optimum pH and temperature for the endoxylanase activity were studied on crude extracts obtained in the semisolid fermentation. Endoxylanase was active between pH 4.0 and 8.4, showing an optimum value at 6.0 (Fig. 7A). The enzyme exhibited about 65% of its activity at pH 8.4.

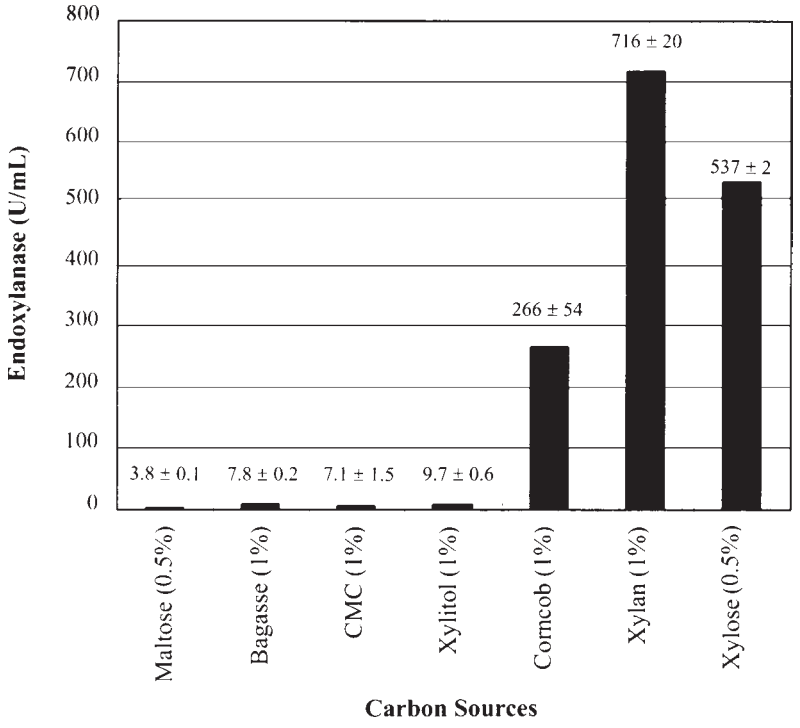

Fig. 5. Production of endoxylanase by *T. lanuginosus* IOC-4145 in liquid medium using different carbon sources after 48 h of cultivation utilizing 10% (v/v) of preinoculum. The enzymatic activity was assayed in 1% xylan in Universal buffer 0.12 *M* at 75°C for 3 min.

Table 2
Values Obtained of Protein and Specific Activity of Crude Extract from *T. lanuginosus* IOC-4145

Substrate	Protein (mg/mL)[a]	Specific activity (U/mg)[b]
κ-Carrageenan (1%)	4.4 ± 0.1	ND
Cashew gum (1%)	6.7 ± 0.6	ND
Pinus gum (1%)	5.9 ± 0.7	ND
Sugarcane bagasse (1%)	7.1 ± 0.4	1.1 ± 0.1
Carboxymethylcellulose (1%)	5.1 ± 0.7	1.5 ± 0.5
Xylitol (1%)	5.2 ± 0.3	1.9 ± 0.0
Corncob (1%)	6.5 ± 0.2	50 ± 9
Xylan (1%)	5.9 ± 1.1	126 ± 20
Xylose (0.5%)	8.1 ± 0.4	67 ± 4
Maltose (0.5%)	5.5 ± 0.1	0.7 ± 0.0
Fructose (0.5%)	5.9 ± 0.1	ND
Glucose (0.5%)	5.2 ± 0.2	ND

[a]Protein of the extracellular enzymatic extract.
[b]ND, not determined.

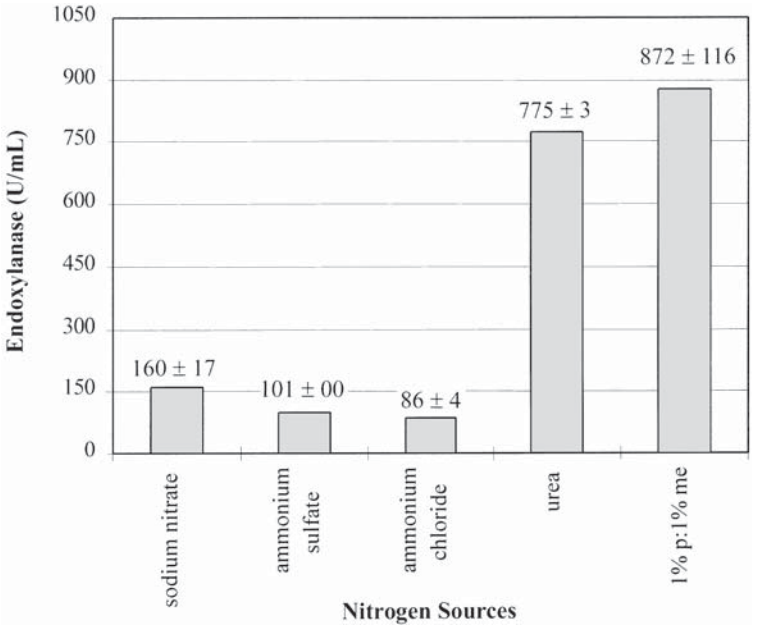

Fig. 6. Production of endoxylanase by *T. lanuginosus* IOC-4145 in semisolid cultivation using different nitrogen sources after 72 h of cultivation utilizing 20% (v/v) of preinoculum. The enzymatic activity was assayed in 1% xylan in Universal buffer 0.12 *M* at 75°C for 3 min. p, peptone; me, meat extract.

However, a rapid inactivation of endoxylanase was observed at pH 9.0 and above.

The temperature for maximum activity was estimated to be 75°C (Fig. 7B). Endoxylanase was shown to be stable over a broad range of temperatures (50–85°C), retaining 60% of the initial rate at 85°C.

It was found that the enzyme was stable in the pH range of 5.0–10.0 after 90 h of incubation at 35°C. At pH 4.0 the enzymes retained >70% of the initial rate (data not shown).

The investigation of the thermal stability of endoxylanase revealed that the enzyme was very stable at 50°C for 6 h of incubation, showing a half-life of 24 h; at 60°C the enzyme retained 50% of the initial activity after 4 h of incubation, whereas at 70°C, more than 80% of activity was lost after 1 h (Fig. 8). Storage of the crude culture filtrate at –20°C for 6 mo showed a small loss (15–20%) from initial activity.

The high temperature (75°C) and pH (6.0) optimum values and thermal stability, as well as optimum activities at alkaline pH values for endoxylanase of *T. lanuginosus* IOC-4145, reflect attractive properties for use on an industrial scale. It is therefore doubtless that more stable, and in particular more thermostable, xylanases would be extremely useful in the pulp and paper industry as bleaching boosters. Thermostable enzymes can be conveniently stored, handled, and transported at room temperatures and could be more effectively recycled.

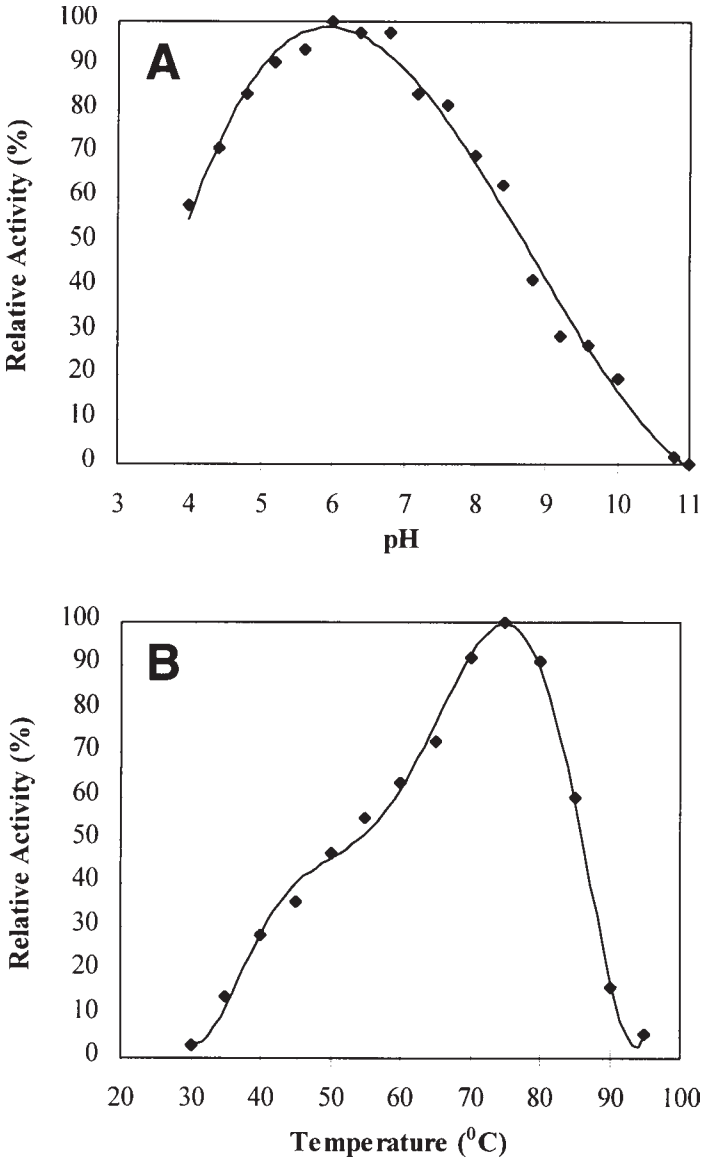

Fig. 7. Effects of pH **(A)** and temperature **(B)** on the initial velocity of endoxylanase of *T. lanuginosus* IOC-4145. The enzymatic activity was assay in 1% xylan at different pH and temperatures for 3 min.

Conclusion

The physicochemical and enzymatic properties of *T. lanuginosus* IOC-4145 endoxylanase studied, as well as the mode of action of its production using the semisolid fermentation with low-cost lignocellulosic material, suggest that the enzyme could provide a valuable tool for a number of technological and biotechnological applications.

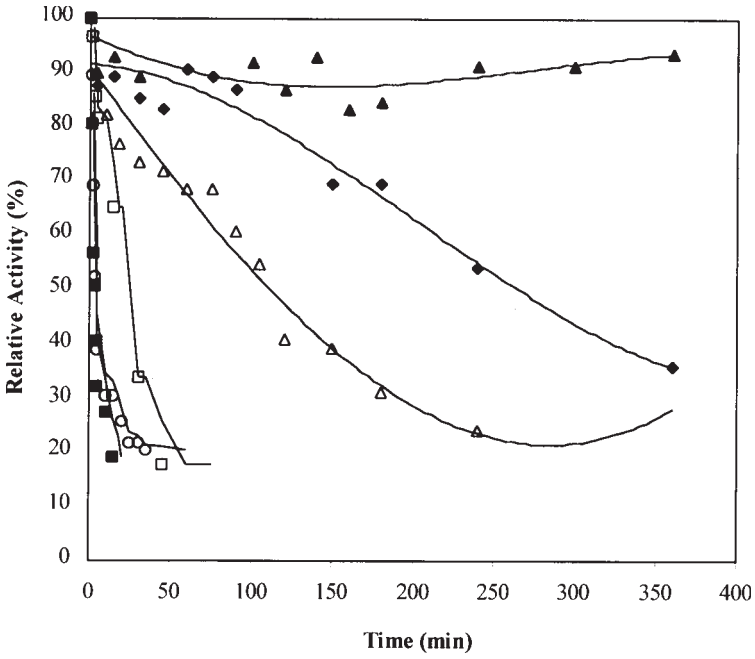

Fig. 8. Thermostability of endoxylanase from *T. lanuginosus* IOC-4145. (—▲—), 50°C; (—◆—), 60°C; (—△—), 65°C; (—□—), 70°C; (—○—), 80°C; (—■—), 90°C. The enzymatic activity was assayed in 1% xylan in Universal buffer 0.12 *M* at 75°C for 3 min.

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