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Application of Xylanase from *Thermomyces lanuginosus* IOC-4145 for Enzymatic Hydrolysis of Corncob and Sugarcane Bagasse

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Abstract

Xylanases have significant current and potential uses for several industries including paper and pulp, food, and biofuel. For the biofuel industry, xylanases can be used to aid in the conversion of lignocellulose to fermentable sugars (e.g., xylose). We investigated the thermophilic fungus *Thermomyces lanuginosus* was yielded for xylanase production and found that the highest activity (850 U/mL) was yielded after 96 h of semisolid fermentation. The enzyme was used for hydrolyzing agricultural residues with and without pretreatment. Such residues were characterized in relation to the maximum xylose content by total acid hydrolysis. The highest xylose yields realized by enzymatic hydrolysis were 24 and 52%, achieved by using 3000 U/g (dried material) of sugarcane bagasse and corncob, respectively, which received both alkali and thermal pretreatment.

Index Entries: Xylanase; *Thermomyces lanuginosus*; agricultural residues; enzymatic hydrolysis; corncob; sugarcane bagasse.

Introduction

Hemicellulases, similar to the cellulase complex, are seldom found in isolation but are usually present as part of a multicomponent system.

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The complete degradation of hemicellulose becomes more complex than that of cellulase, since substituent-hydrolyzing activities are also necessary. With heteroxylans, apart from endo-1,4-β-xylanase, which catalyzes the hydrolysis of internal β-1,4-xylan links and β-xylosidase, which catalyzes the hydrolysis of xylooligossacharides, mainly xylobiose into xylose, other enzymes must act to accomplish complete hydrolysis, such as acetyl xylan esterase, α-glucuronidase, and α-L-arabinofuranosidase *(1)*.

Xylanases have a wide range of potential biotechnological applications. They catalyze the hydrolysis of internal β-1,4-D-xylose units and are used in bread making, in recovery of textile fibers, in clarification of beer and juices, and in hydrolysis of xylan-containing lignocellulosic materials to D-xylose, which can be converted to a variety of bioproducts with high aggregate value *(1–3)*. In addition, there has been increasing interest in using thermostable cellulase-free xylanases in the paper and pulp industry to modify pulp properties by removing hemicelluloses without disturbing the cellulose fiber strength of paper products *(4,5)*.

Xylanases are produced by a wide variety of microorganisms, and among them the fungi are the most potent producers. The *Thermomyces lanuginosus* strain IOC-4145, isolated from Brazilian soil, secreted cellulase-free xylanase activity in submerged and semisolid fermentation using corncob as substrate *(6)*. Some properties of the crude enzyme preparation have been published *(6,7)*, and the efficient use of this thermophilic preparation in the bleaching of acetosolv *(8)* and ethanol/water *(9)* bagasse pulps has also been reported.

There are very few articles reporting the use of xylanase in enzymatic hydrolysis of xylan-containing raw materials to obtain xylose *(10,11)*. Xylose is a fermentable sugar with high market price. Thus, the utilization of enzymatic hydrolysis to obtain xylose from agricultural residues is of great interest in modern biotechnology, because it is a cleaner and more specific procedure compared with the conventional technology to produce this abundant pentose.

In this article, we describe the production of xylanase by *T. lanuginosus* IOC-4145 in semisolid cultivation using corncob as raw material in optimized conditions. Furthermore, we describe the pretreatment effect on corncob and sugarcane bagasse and the enzymatic hydrolysis of these lignocellulosic materials using the produced thermophilic xylanase.

Materials and Methods

Chemicals

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

Organism and Growth Conditions

The strain of *T. lanuginosus* used was isolated from soil at IBILCE/UNESP/SP, Brazil, and identified by Fundação Instituto Oswaldo Cruz

(Rio de Janeiro, Brazil), under the code IOC-4145. This strain was maintained on slants of oat agar: 50.0 g/L of oats, and 30.0 g/L of agar at 4°C. The composition of the growth medium (GPMKC) was as follows: 10.0 g/L of peptone, 10.0; 10.0 g/L of meat extract, 10.0 g/L of NaCl, 1.0 g/L of KH_2PO_4, 15.0 g/L of glucose. The production medium (PPMKC) had the following composition: 2.0 g/L of peptone, 2.0 g/L of meat extract, 2.0 g/L of NaCl, and 1.0 g/L of KH_2PO_4. The semisolid medium was composed of 15 g of corncob and 22.5 mL of PPMKC medium. The corncob was kindly supplied by Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), Brazil).

Spore and Mycelium Suspensions

Spores suspensions were prepared by adding 3 mL of 0.1% Tween-80 to slant cultures and gently scraping off the surface with a sterilized wire loop. These spores were counted in a Neubauer chamber, and a standardized amount (10^6 spores/mL) was inoculated in the GPMKC medium. The experiments were carried out by inoculating the GPMKC medium (pH6.0) with a mycelium suspension, germinated previously for 20 h at 45°C and 150 rpm.

Xylanase Production in Semisolid Fermentation

Twenty percent (v/v) mycelium suspension was used to inoculate 500-mL conical flasks containing 15 g of corncob as carbon source and 22.5 mL of PPMKC medium (pH6.0) as optimized by Damaso et al. *(6)*. After inoculation, the flasks were incubated in a stationary manner at 45°C for 6 d in a laboratory electric incubator. At each sampling time, the culture medium was vacuum filtered using filter paper (Whatman, no. 4, fast-flow rate), and the filtrate was used for further enzyme assays. During the cultivation, two or more flasks were sampled daily.

Enzyme Assays

Xylanase was assayed using birchwood xylan as substrate. The solution of xylan and the enzyme at appropriated dilution were incubated at 75°C for 3 min, and the reducing sugar was determined by the dinitrosalicylic acid procedure *(12)* with xylose as standard. The released color development was measured spectrophotometrically at 540 nm. One unit of enzyme activity was defined as 1 μmol of reducing sugar released 1 min under the described assay conditions. Protein concentration was measured by the Lowry method *(13)* using bovine serum albumin as standard.

All experiments were performed in duplicate and the analytical measurements at least in triplicate.

Acid Hydrolysis of Corncob and Sugarcane Bagasse

Samples of 1 g of lignocellulosic material were weighed and placed in 100-ml beakers. This material was treated with 5 mL of 72% H_2SO_4 and continuously stirred with a glass rod at 45°C for 7 min. The reaction was

stopped with the addition of 50 mL of distilled water. Then, the samples were transferred to 250-mL conical flasks, and water was added to a total volume of 137 mL. For total hydrolysis, the flasks were closed and treated thermally for 30 min at 121°C. The reaction mixtures were then filtered using filter paper, and the hydrolysates were transferred to 250-mL volumetric flasks and completed with distilled water *(14)*.

A 20-mL aliquot of hydrolysate was diluted with distilled water in a 25-mLvolumetric flask after the pH of the solution was increased from 0.6 to 5.0 with 2 *N* NaOH. The hydrolysate samples were filtered and checked for xylose yield by high-performance liquid chromatography (HPLC).

Enzymatic Hydrolysis of Corncob and Sugarcane Bagasse

The enzymatic hydrolysis of agricultural substrates was carried out as follows: Corncob, already dried (supplied by EMBRAPA), and sugar cane bagasse (supplied by Usina São Martinho, São Paulo, Brazil), dried in an oven at 60°C for 24 h, were milled in a mill of knives model 6010, at 15 A, and 15 V; Primelétrica LTDA, Brazil to powder form and sieved (42 mesh size). These lignocellulosic materials were not pretreated or were pretreated thermally or pretreated both chemically and thermally. The thermal pretreatment was carried out using approx 20 g of lignocellulosic material and 450 mL of water, for 90 min at 121°C, while the chemical pretreatment was carried out with alkali at similar conditions, using 2 *N* NaOH instead of water, at 30°C for 20 h. The alkali-pretreated samples were washed with distilled water followed by separation to remove the alkali. The lignocellulosic substrate pretreated or not (1.25 g dry wt) was placed into water or universal buffer (0.04 M H_2PO_4, 0.04 *M* CH_3COOH, and 0.04 *M* H_2BO_3 adjusted to pH6.0 with 0.2 *N* NaOH) or phosphate buffer (0.2 *M* NaH_2PO_4 and 0.2 *M* Na_2HPO_4 adjusted to pH6.0) in 500-mL conical flasks, and a desired amount of crude xylanase was added to start the reaction, for a total volume of 35 mL. The hydrolysis reaction mixture was kept under constant shaking at 120 rpm (model G-25, New Brunswick Scientific Co., Edison, NJ); at 40°C, and a control reaction was carried out at the same conditions, substituting the active enzyme with its denaturated form. The hydrolysate samples were withdrawn after different incubation times, filtered, and checked for xylose measurements by HPLC.

Enzymatic Hydrolysis of Xylan

The enzymatic hydrolysis of soluble birchwood xylan was carried out as follows: The substrate (0.5 g) was placed universal or phosphate buffer in 500 mL conical flasks, and 1500 U of crude xylanase was added to start the reaction, for a total volume of 25 mL. The hydrolysis and control reactions were accomplished at the same conditions described for enzymatic hydrolysis of lignocellulosic materials.

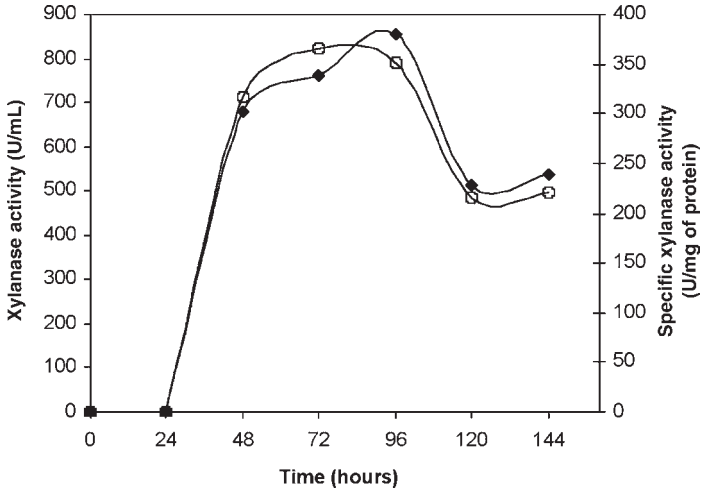

Fig. 1. Kinetic profile of xylanase production (—◆—) by *T. lanuginosus* IOC-4145 and its specific activity (—○—) using corncob as sbustrate on semisolid fermentation. The values correspond to the average of five and three measurements, respectively, and the error was –10%.

End Product Analysis by HPLC

The end products of acid and enzymatic hydrolysis of corncob and sugarcane bagasse were analyzed by using a high-performance liquid chromatograph (Waters, Milford, MA) equipped with a Rheodyne automatic injector with a 20-µL injection capacity loop, a Shodex Sugar SC 1011 column, and an integrator model 747 with a model 410 RI detector. The mobile phase was deionized water, and the flow rate was adjusted to 0.8 mL/min.

Results and Discussion

Kinetics of Xylanase Production in Semisolid Fermentation

The kinetic profile of the enzymatic production by *T. lanuginosus* IOC-4145 in semisolid medium using corncob as substrate was obtained after cultivation at 45°C for 144 h. The maximum peaks of xylanase production and specific activity occurred after 96 and 72 h of cultivation, respectively (Fig. 1). Note that most of the protein produced is related to the xylanase secreted by the fungus. High activities (850 U/mL and 365 U/mg of protein) were obtained using corncob, denoting that this residue is a good substrate for xylanase production by *T. lanuginosus* IOC-4145. When compared with other data reported in the literature employing the same or different lignocellulosic materials and other microorganisms *(15–17)*, the strain used in the present work proved to be a promising microorganism for xylanase production. The production of xylanases by *Aspergillus niger* on semisolid cultures supplemented with wheat bran revealed a

Table 1
Xylose Yield of Corncob and Sugarcane Bagasse With and Without Pretreatment

	Xylose yield (% w/w)[a]	
Pretreatment	Corncob	Sugarcane bagasse
None	22.2 ± 3.5	20.3 ± 2.4
Thermal	18.2 ± 2.1	16.8 ± 1.4
Alkali and thermal	12.2 ± 1.8	10.4 ± 0.9

[a] On dry basis.

xylanase level of 40 U/mL *(16)*, and that reached by *Aspergillus terreus* in semisolid fermentation led to an enzyme level of about 22 U/mL *(15)*.

Determination of Xylose in Sugarcane Bagasse and Corncob by Acid Hydrolysis

Acid hydrolysis was used to determine the amount of xylose present in the lignocellulosic materials without pretreatment, after thermal pretreatment, and after both alkali and thermal pretreatments.

Table 1 shows the percentage of xylose present in corncob and sugarcane bagasse with and without pretreatments. The values are in accordance with those reported in the literature *(18)*. The results also revealed that xylose content decreased when pretreatments were applied (alkali and thermal), particularly when both of them were used.

Enzymatic Hydrolysis of Xylan, Sugarcane Bagasse, and Corncob With and Without Pretreatments

Enzymatic hydrolysis of sugarcane bagasse and corncob was carried out with a concentration of solids of 36 g of biomass TL using different amounts of xylanase as well as different incubation times and conditions of the reaction.

Table 2 depicts the results of the enzymatic hydrolysis of sugarcane bagasse after thermal pretreatment with different enzyme concentrations (1000, 2000, 3000, 4000, and 5000 U/g of dried material) using universal buffer. Hydrolysis was carried out with different intervals of time, and the samples were analyzed to evaluate the degree of hydrolysis. In the initial times of hydrolysis (2 and 6 h of incubation), lower values of the degree of hydrolysis were obtained, independently of the enzyme concentration. On the other hand, after 24 h of incubation, the degree of hydrolysis increased and the best result was achieved with 3000 U/g of dried material, decreasing with higher enzyme concentration (4000 and 5000 U/g of dried material), which might be owing to any steric hindrance effect.

The second set of experiments was carried out using sugarcane bagasse and corncob both pretreated either thermally or with alkali and thermally, using a fixed amount of enzyme (3000 U/g of dried material).

Table 2
Degree of Hydrolysis of Thermally Pretreated Sugarcane Bagasse After Incubation with Xylanase from *T. lanuginosus* IOC-4145

Total enzyme (U)/g of dried material	Relative degree of hydrolysis (%) [a]		
	2 h	6 h	24 h
1000	11.0	12.3	19.4
2000	9.6	12.1	47.2
3000	12.7	11.3	100.0
4000	8.7	11.2	50.6
5000	12.0	6.2	57.4

[a] The degree is expressed as the percentage of the maximum hydrolysis obtained with 3000 U of xylanase g of dried material after 24 h of incubation. The values correspond to the average of two measurements and the error was 12.5%.

Table 3
Effect of Enzymatic Hydrolysis Using 3000 U of Xylanase from *T. lanuginosus* IOC-4145 g of Sugarcane Bagasse Under Different Conditions After 24 h of Incubation

Condition [a]	Xylose yield (mg/g of bagasse) [b]	Degree of hydrolysis (%)[b]
1 (A, T, W)	1.4	1.4
2 (A, T, PB)	25.2	24.3
3 (A, T, UB)	19.6	18.9
4 (T, W)	0.0	0.0
5 (T, PB)	0.4	0.2
6 (T, UB)	4.4	2.6

[a] A, T, W: alkali and thermal pretreatments and the presence of water; A, T, PB: alkali and thermal pretreatments and the presence of phosphate buffer; A, T, UB: alkali and thermal pretreatments and the presence of universal buffer; T, W: thermal pretreatment and the presence of water; T, PB: thermal pretreatment and the presence of phosphate buffer; T, UB: thermal pretreatment and the presence of universal buffer.

[b] The values correspond to the average of two measurements and the error was <15%.

In addition, the effect of water, and universal and phosphate buffer, on the degree of hydrolysis was investigated (Tables 3 and 4). It can be observed that it was necessary to use both pretreatments (conditions 1, 2, and 3) to obtain higher amounts of xylose. Probably, this could be ascribed to the fact that these pretreatments had opened up the pores of the cell wall structure, enhancing the enzyme's access to the substrate

Table 4
Effect of Enzymatic Hydrolysis Using 3000 U of Xylanase from *T. lanuginosus* IOC-4145/g of Corncob Under Different Conditions After 24 h of Incubation

Condition[a]	Xylose yield (mg/g of corncob)[b]	Degree of hydrolysis (%)[b]
1 (A, T, W)	25.0	20.5
2 (A, T, PB)	33.4	27.3
3 (A, T, UB)	63.4	51.9
4 (T, W)	0.5	0.3
5 (T, PB)	0.6	0.4
6 (T, UB)	0.8	0.5

[a]A, T, W: alkali and thermal pretreatments and the presence of water; A, T, PB: alkali and thermal pretreatments and the presence of phosphate buffer; A, T, UB: alkali and thermal pretreatments and the presence of universal buffer; T, W: thermal pretreatment and the presence of water; T, PB: thermal pretreatment and the presence of phosphate buffer; T, UB: thermal pretreatment and the presence of universal buffer.

[b]The values correspond to the average of two measurements and the error was <12.8%.

and, consequently, improving the hydrolysis yield. Additionally, the presence of buffer during the enzymatic hydrolysis (conditions 2 and 3), instead of simple addition of water (condition 1), was fundamental for higher xylanase efficiency.

Finally, the best condition for enzymatic hydrolysis was attained using corncob treated both with alkali and thermally in medium containing the universal buffer, achieving a degree of hydrolysis of 52%, which corresponds to 63.4 mg of xylose per gram of dried corncob. Comparison of these results with those obtained with enzymatic hydrolysis of soluble substrate xylan (Fig. 2), in which the best results (107 mg of xylose g of xylan and degree of hydrolysis of 21%) were also obtained using universal buffer, shows that after 24 h of incubation, the efficiency of xylanase in pretreated corncob was corroborated (Table 4).

Christov and Prior *(10)* used a crude xylanase preparation from *Aurebasidium pullulans* to remove hemicellulose from unbleached sulfite pulp in which the pulp was pretreated with 0.03 g of NaOH 1 h of pulp at 80°C for 1 h and with 2.5% pulp consistency. After 24 h of incubation, 12.8 mg of reducing sugar 1 g of pulp was produced, fivefold lower than the value achieved in the present work (63.4 mg of xylose 1 g of dried corncob), although different pretreatment conditions had been employed.

Gokhale et al. *(11)* studied the application of a yeast cellulase-free xylanase in agrowaste materials, such as bleached bagasse pulp, jute fiber, and corncob powder. The best result was achieved using bleached bagasse pulp with a degree of hydrolysis of 19.4%.

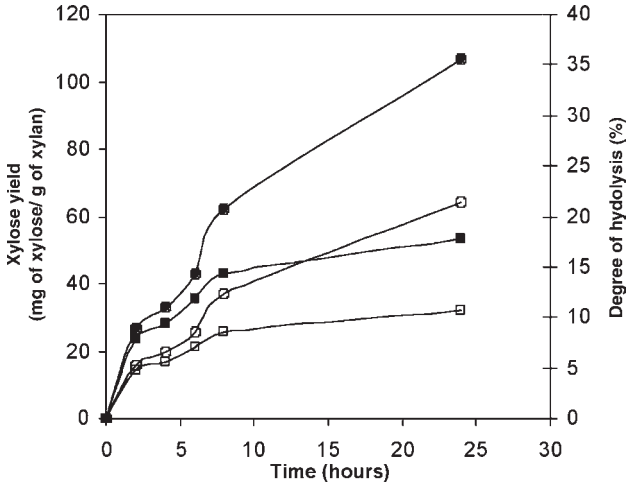

Fig. 2. Influence of phosphate and universal buffers in enzymatic hydrolysis of soluble birchwood xylan (Sigma) using 3000 U of xylanase from *T. lanuginosus* IOC-4145/g of substrate: (●) xylose yield–universal buffer; (○) degree of hydrolysis–universal buffer; (■) xylose yield–phosphate buffer; (□) degree of hydrolysis–phosphate buffer. The values correspond to the average of two measurements, and the error was <9.5%.

In conclusion, although the accessory enzyme activities needed to complete the degradation of the hemicellulose fraction in the crude extract were not all determined it was evident that the *T. lanuginosus* was capable of producing them. This novel, isolated, filamentous fungus has shown the capacity to produce appreciable amounts of xylose from corncob and sugarcane bagasse, which are abundant residues from the agroindustry. These residues have no production costs attached to them and are environmental polluters. Their enzymatic hydrolysis is a clean technology, of innovative approach and poorly exploited, needing further investigation in order to improve the xylose yield, and consequently the degree of hydrolysis.

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