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Production and Characterization of Cellulase-Free Xylanase from *Trichoderma inhamatum*

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Abstract The production of extracellular cellulase-free xylanase from *Trichoderma inhamatum* was evaluated in liquid Vogel medium with different carbon sources as natural substrates and agricultural or agro-industrial wastes. Optimal production of 244.02 U/mL was obtained with xylan as carbon source, pH 6.0 at 25°C, 120 rpm, and 60-h time culture. Optimal conditions for enzyme activity were 50°C and pH 5.5. Thermal stability of *T. inhamatum* xylanolytic complex expressed as $T_{1/2}$ was 2.2 h at 40°C and 2 min at 50°C. The pH stability was high from 4.0 to 11.0. These results indicate possible employment of such enzymatic complex in some industrial processes which require activity in acid pH, wide-ranging pH stability, and cellulase activity absence.

Keywords Xylanase · *Trichoderma inhamatum* · Optimization · Enzyme characterization · Enzyme regulation

Introduction

The most abundant and renewable biomass available on earth is lignocellulose, which contains three major groups of polymers: cellulose, hemicellulose, and lignin [1, 2]. Xylan, the most plentiful hemicellulose, is, after cellulose, the most abundant polysaccharides in nature. It is part of the plant cell walls of hardwoods and softwoods, of all graminaceous plants, and is particularly abundant in tissues that have undergone secondary thickening [2, 3]. Then, it is the most abundant hemicellulose in agricultural and several agro-industrial wastes.

Xylan is an heteropolysaccharide with an homopolymeric backbone chain of β-1,4 linked D-xylopyranose units. The backbone is commonly substituted with L-arabinofuranose and D-glucuronic or 4-*O*-metyl-D-glucuronic acid. The xylan can be also esterified by acetic acid at position 2 and/or 3 of xylopyranosyl units and by ferulic or *p*-coumaric acid at position 5 of arabinofuranosyl units [4, 5]. Because of the heterogeneity of xylan, their complete hydrolysis requires the action of a complex enzymatic system.

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The best known xylanolytic enzymes are endo-β-1,4-xylanases (EC 3.2.1.8), which act on the chain of xylan, cleaving the internal glycosidic linkages of the backbone that results in chains with lower polymerization degree and β-xylosidase (EC 3.2.1.37), which hydrolyses xylobiose and short xylooligosaccharides to xylose [6–9]. Xylanases are produced by both prokaryotes and eukaryotes. A large number of bacteria and fungi are known as xylanase producers. From an industrial point of view, filamentous fungi are interesting producers of these enzymes due to xylanases releasing [4, 6].

Microbial xylanases is a group of industrial enzymes with applications in animal feed, pulp and paper bleaching, textile, and food processing. In some of these applications, a specific preparation cellulase-free is required to maintain, as much as possible, the integrity of the pulps or fibers, e.g., for cellulose pulps preparation and textile fibers liberation [8, 9–15]. One of the major sources of industrial hydrolytic enzymes is the genus *Trichoderma* [16].

Among the 80 strains isolated from Atlantic forest soil, *Trichoderma inhamatum* attracted attention due to its highest xylanolytic activity [17]. This work aimed to determine the influence of physical and chemical factors on xylanase production as well as the xylanolytic complex characterization excreted by this fungus.

Materials and Methods

Organism and Growth

T. inhamatum used in the present work was from Culture Collection of Environmental Studies Center—CEA/UNESP. It was maintained on slants of solid Vogel medium [18] with wheat bran as carbon source. Spores of *T. inhamatum* were obtained from 7 days cultures in the same medium containing 1% (*w*/*v*) glucose. Liquid cultures were prepared in this medium with 1% (*w*/*v*) of the carbon source mentioned, in adjusted or buffered pH for each experiment. Erlenmeyer flasks (125 mL) containing 25 mL of the medium were inoculated with 1.0 mL of spore suspension (10^7 spores/mL) and, when unspecified, they were incubated at 25°C for 60 h under shaking at 120 rpm. All cultures were developed in triplicate and the results presented through mean values.

Enzyme Preparations and Assays

Cultures were harvested by filtration. The filtrate was assayed for extracellular activity and protein. The mycelium was washed with distilled and sterilized water, frozen, and ground with sand in 50 mM sodium phosphate buffer pH 6.0. The slurry was centrifuged at 3,900 *g* at 4°C and the supernatant was the intracellular enzymes source. For determination of total dry mass, mycelial cells were harvested by vacuum filtration, transferred to preweighed porcelain crucible, and dried at 100°C to constant mass.

Xylanase activity was determined at 50°C using 1.0% (*w*/*v*) birchwood xylan (Sigma) in 50 mM sodium phosphate buffer pH 6.0. The reducing sugars released were determined by the dinitrosalicylic acid method [19]. One unit of enzyme activity was defined as the amount of enzyme, which releases 1 μmol of reducing sugar equivalent to xylose per minute.

Avicelase and carboxymethylcellulase (CMCase) activities were determined through the same method using avicel (Riedel de Häen) and carboxymethylcellulose (CMC; Fluka A. G.) 1.0% (*w*/*v*) as substrate. Standard cellulolytic assay conditions were 45°C and pH 5.0

buffered with 50 mM sodium acetate. One unit of cellulase activity was defined as the amount of enzyme that releases 1.0 μmol of reducing sugar equivalent to glucose per minutes. In all cases, specific activities were expressed as activity units per milligram of extracellular protein.

Protein Determination

Protein content was measured by Lowry method [20] using bovine serum albumin as standard.

Enzyme Production on Various Substrates

To select the suitable carbon source for the enzyme production, *T. inhamatum* strain was grown in 125 mL Erlenmeyer flask containing 1% of each substrate (avicel, celobiose, CMC, glucose, lactose, maltose, sucrose, xylan, xylose, sugar cane bagasse, oat bran, wheat bran, rice straw, corncob, and citrus pectin). After 5 days at 28°C and pH 6.5 under standing culture, the enzyme was harvested and assayed.

Effect of Culture Conditions, pH, and Temperature on Xylanase Production

The culture conditions influence on incubation period was studied under standing culture during 12 days and under shaking culture of 120 rpm during 132 h (5.5 days). The effect of initial pH on the enzyme production was analyzed from 3.0 to 9.0 and the temperature influence was verified from 15°C to 40°C.

Enzyme Characterization

Optimum pH and Temperature for Xylanolytic Complex Activity

Enzyme activity was determined at different pH values using McIlvaine buffer from 3.0 to 8.0, at 50°C. The optimum temperature was assayed in the optimum pH by the incubation of xylanolytic complex in a range from 25°C to 60°C.

Stability of Xylanolytic Complex at Different Temperatures and pHs

The crude enzyme diluted (1:1) in McIlvaine buffer (pH 3.0 to 8.0) or glycine–NaOH (pH 8.5 to 11.0) was maintained at 25°C for 24 h, and then the remaining activity was determined. The crude extract was incubated at 40°C, 50°C, and 60°C in optimum pH for xylanolytic complex thermostability determination.

Results and Discussion

Influence of the Carbon Source on Xylanase Production

The effect of several carbon sources on the xylanase production was investigated using different substrates, as pure carbohydrate (Table 1) and some natural substrates (Table 2). The highest levels of both intracellular and extracellular xylanase activities were detected in medium supplemented with xylan. In all cases, the values of intracellular activity were much lower than those of the extracellular activity. Among the pure carbohydrates, xylan

Table 1 Influence of some pure carbohydrates on xylanase production by *T. inhamatum*.

Carbon source	Enzymatic activity (U/mL)	Specific activity (U/mg protein)
Glucose	0.25±0.02	0.35±0.02
Xylose	2.66±0.17	0.58±0.03
Maltose	ND	ND
Lactose	2.68±0.02	3.13±0.06
Sucrose	ND	ND
Celobiose	0.10±0.01	0.10±0.00
Avicel	0.19±0.00	0.11±0.00
CMC	0.21±0.00	0.12±0.00
Xylan	50.01±1.04	20.34±0.29

Average and standard deviation of three cultures

ND Not detectable

showed to be the best inducer, followed by lactose and xylose, as for units of activity per volume and for specific activity as well. Media with celobiose, avicel, CMC, and glucose exhibited xylanase activity much lower than that with xylan. These results indicate that in this microorganism as in many others, the xylanase activity is inducible and that xylan and related substrates have an important role in its induction [4]. *T. inhamatum* xylanase synthesis can be also affected by carbon catabolite repression, as verified in other filamentous fungi. Enzyme activity was not detected with maltose and sucrose as carbon source (Table 1), showing that *T. inhamatum* xylanase is noninducible with these disaccharides. In *Streptomyces* sp. [21], *Trichosporon cutaneum* [22], *Clostridium absonum* [23], *Thermomyces lanuginous* [24], *Streptomyces* sp. [25], and *Aspergillus giganteus* [26] xylan and xylose induced the highest levels of xylanase activity.

The xylanase production by *T. inhamatum* with different agricultural and agro-industrial wastes showed the highest values of activity per volume (Table 2) in media containing wheat bran, corn cob, oat bran, and sugar cane bagasse, respectively. Due to the variation of the extracellular protein concentration, the order of specific activity was sugar cane bagasse, wheat bran, oat bran, and corn cob. These results indicate that wheat bran, oat bran, and corn cob can be used to produce xylanase by this fungus.

In *T. reesei* Rut C-30, significant levels of xylanase activity were obtained in cultures on L-arabinose-rich plant products, as oat husk and sugar beet pulp hydrolysates [16]. Canola meal as a substrate showed to be an efficient inducer for the xylanase production by *T. reesei* [27]. Seyis and Aksoz [28] studying some natural wastes to produce xylanase from *T. harzianum* 1073 D3 verified that melon peel exhibited the maximum activity. In liquid

Table 2 Effect of different agricultural and agro-industrial wastes on xylanase production by *T. inhamatum*.

Carbon source	Enzymatic activity (U/mL)	Specific activity (U/mg protein)
Sugar cane bagasse	3.38±0.32	4.00±0.12
Wheat bran	11.37±1.01	3.46±0.15
Oat bran	10.11±0.27	2.64±0.05
Rice straw	0.30±0.02	0.25±0.03
Pectin of citrus	0.34±0.03	0.16±0.01
Corn cob	10.41±0.29	2.63±0.10

Average and standard deviation of three cultures

cultures of *A. versicolor*, wheat bran induced very similar values of xylanolytic activity to that of xylan [29].

The carbon source used in the subsequent experiments was oat spelt xylan. Considering that some xylanase applications demand absence of cellulolytic activity and that the cellulase make difficult the xylanase purification, the filtrates were assayed for avicelase and CMCase activities. In all cases, the cellulolytic activities were not detected under the assays conditions.

Effects of Culture Conditions on Xylanase Production

The maxima intracellular and extracellular xylanase production in standing condition were obtained with 96 h or 4 days of cultivation at 28°C using xylan as carbon source (Fig. 1a). In shaking condition (Fig. 1b), those maxima were observed only at 60 h or 2.5 days at 28°C. Extracellular xylanase activity values were 98.99 and 102.94 U/mL and for specific activity were 34.37 and 38.36 U/mg of protein in these conditions, respectively. The values obtained are not significantly different, in opposition to those verified by Coelho and Carmona of 57.1 and 32.5 U/ml for activity and 96.4 and 66.4 U/mg of protein for specific activity, in both conditions, respectively [26]. These authors observed peaks for *A. giganteus* xylanase activity in 5.0 days and 3.5 days culture under standing and shaking condition with xylan as carbon

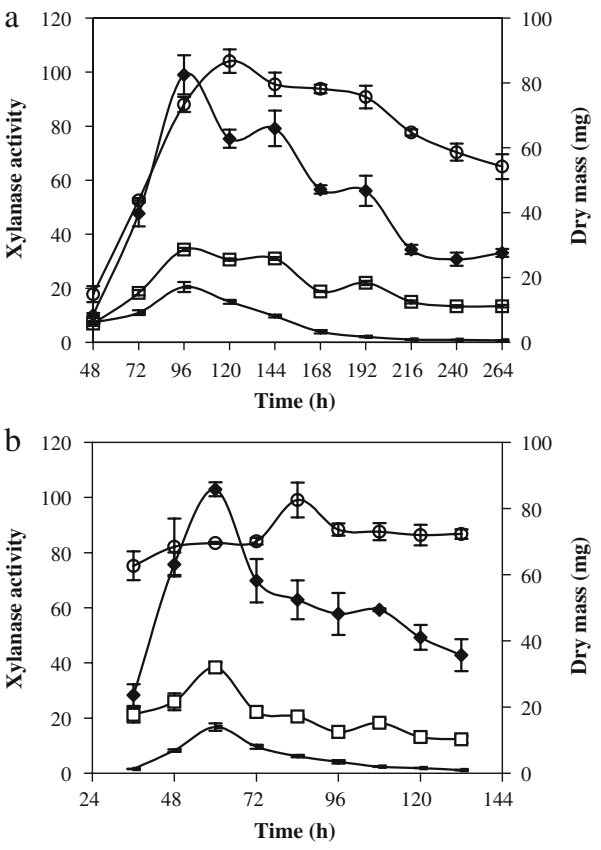

Fig. 1 Time course of xylanase production by *T. inhamatum* in **a** under standing condition and **b** shaking at 120 rpm. Culture condition—Vogel medium containing xylan 1% (*w*/*v*), 28°C, and pH 6.5; (*filled diamond*) extracellular xylanase (U/mL), (–) intracellular xylanase (U/mL), (*unfilled square*) specific xylanase activity (U/mg of protein), and (*unfilled circle*) dry mass (mg)

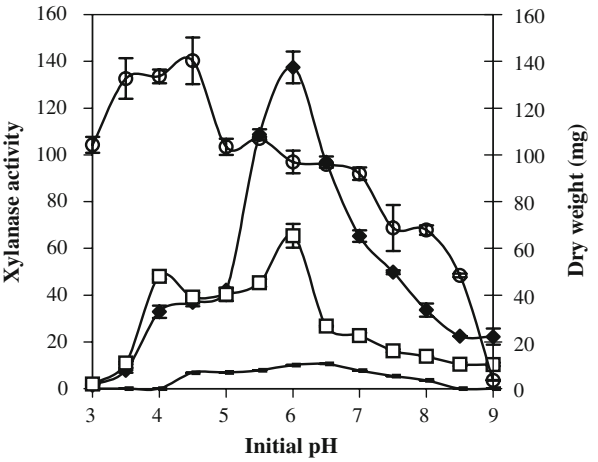

Fig. 2 Culture initial pH effect on xylanase production by *T. inhamatum*. Culture conditions—Vogel medium with xylan 1% (*w*/*v*) shaking at 120 rpm and 28°C; (*filled diamond*) extracellular xylanase (U/mL), (–) intracellular xylanase (U/mL), (*unfilled square*) specific xylanase activity (U/mg of protein), and (*unfilled circle*) dry mass (mg)

source. Seyis and Aksoz [28], using xylan as the only carbon source for xylanase production from *T. harzianum* 1073 D3 obtained the maximum level of activity at the end of day 13 or 312 h in standing condition.

The highest *T. inhamatum* growth occurred at 120 h or 5 days in standing culture and 84 h or 3.5 days in shaking culture (Fig. 1), indicating that xylanase was expressed during the exponential phase. According to Kulkarni et al. [30], this is usually observed for xylanases and the harvesting time is correlated to the medium under consideration. The subsequent experiments were carried out under shaking of 120 rpm, since the maximum productivity was reached within the shortest period of time (60 h), resulting in lower costs and making this condition more advantageous.

The pH is an important parameter in the production of enzymes, and its influence on xylanase production by *T. inhamatum* is showed in the Fig. 2. The highest values for xylanase activity were observed at initial pH 6.0, corresponding to 137.44 U/mL and 65.23 U/mg of protein. For *T. reesei* Rut C-30 [31], the maximum production of the xylanolytic complex was also obtained at pH 6.0.

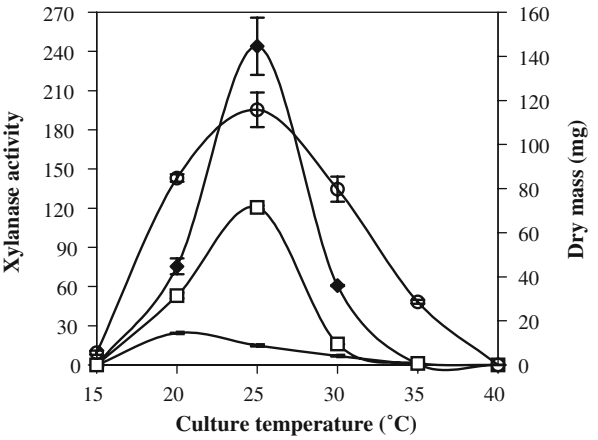

Fig. 3 Temperature culture effect on xylanase production by *T. inhamatum*. Culture conditions—Vogel medium with xylan 1% (*w*/*v*) shaking at 120 rpm and pH 6.0; (*filled diamond*) extracellular xylanase (U/mL), (–) intracellular xylanase (U/mL), (*unfilled square*) specific xylanase activity (U/mg of protein), and (*unfilled circle*) dry mass (mg)

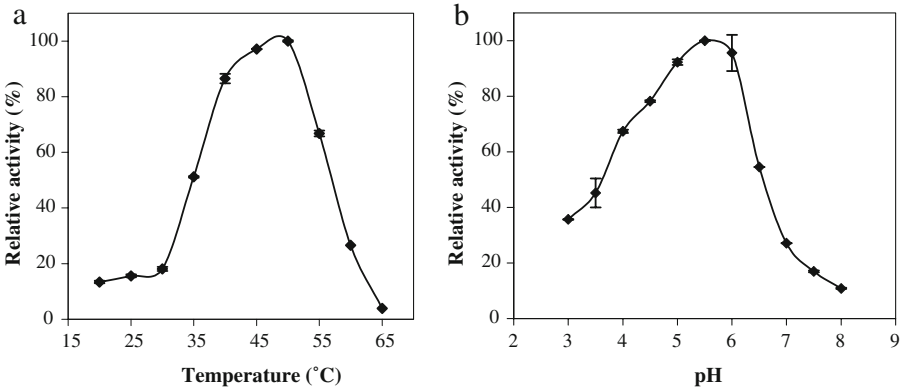

Fig. 4 Temperature (**a**) and pH (**b**) influence on extracellular xylanase activity from *T. inhamatum*. Culture condition—Vogel medium with xylan 1% (*w*/*v*) under shaking conditions at 120 rpm for 60 h pH 6.0. In **a** enzymatic activity was determined in 50 mM sodium phosphate buffer pH 6.0, and in **b** it was determined at 50°C in McIlvaine buffer

T. inhamatum strain grows in different initial pHs from 3.0 to 8.5 (Fig. 2), with maximum in the range of 3.5 to 4.5. This result clearly indicates the acidophilic nature of the fungus.

The temperature effect on xylanase production by *T. inhamatum* is showed in Fig. 3. The optimum temperature of production was observed at 25°C, corresponding to 244.02 U/mL and 120.62 U/mg of protein. At 30°C, the production of extracellular xylanase was drastically decreased. Coelho and Carmona [26] obtained a peak of 57 U/mL for *A. giganteus* xylanase activity at 25°C, pH 6.5 in the same medium with the same substrate, and Carmona et al. [29] obtained a peak for *A. versicolor* at 30°C cultivated during 5 days in Vogel medium with wheat bran at pH 6.5.

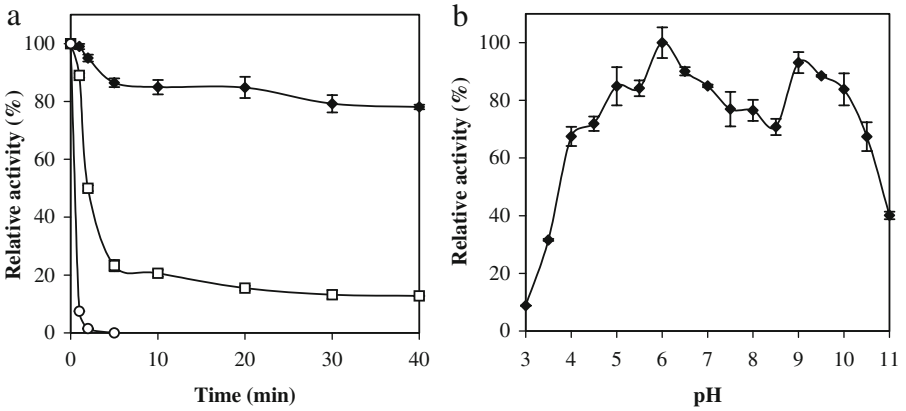

Fig. 5 Thermal (**a**) and pH stabilities (**b**) of the xylanolytic complex from *T. inhamatum*. In **a**, the crude enzyme was heated at (*filled diamond*) 40, (*unfilled square*) 50, and (*unfilled circle*) 60°C temperature without substrate and tested for residual xylanase activity, and in **b**, the crude enzyme was incubated without substrate in McIlvaine buffer (pH 3.0 to 8.0) and glycine–NaOH (pH 8.5 to 11.0) at room temperature for 24 h and assayed for residual xylanase activity

The highest growth determined by mycelial dry mass was verified at 25°C, indicating the mesophilic character of this strain. In all cases, the levels of intracellular xylanase activity were much lower than the extracellular activity.

Properties of Extracellular Crude Xylanase

The profile of xylanase activity from *T. inhamatum* against the temperature (Fig. 4a) showed that the optimum temperature was 50°C. This value is similar to the optimal temperature reported by Medeiros et al. [32] for *T. longibrachiatum* and Coelho and Carmona [26] for *A. giganteus.*

The favorable pH range for xylanase activity of *T. inhamatum* was from 5.0 to 6.0, with optimum at 5.5 (Fig. 4b), but significant activities (above 50%) were detected at 4.0, 4.5, and 6.5, decreasing below 4.0 and above 6.5. This optimal pH falls within the acid region described for most fungal xylanases [24–26, 29].

Besides optima temperature and pH, thermal stability constitutes a very important property for industrial enzymes [27]. The thermal stability of the extracellular crude xylanase from *T. inhamatum* was studied without substrate at 40°C, 50°C, and 60°C (Fig. 5a). The half-life ($T_{1/2}$) at 40°C was 2.2 h and after 6.0 h in this temperature, 25% of the initial activity was remained. At 50°C, $T_{1/2}$ was 2 min and at 60°C, it was even shorter.

The pH stability of the xylanolytic complex of *T. inhamatum* was assayed from 3.0 to 11.0 (Fig. 5b). It showed low stability (below 45%) in pH 3.0, 3.5, and 11.0, high stability (above 60%) in the interval from 4.0 to 10.5 with two peaks, one at 6.0, and the other at 9.0.

This is the first report about the production of extracellular xylanase from *T. inhamatum*. In optimal culture conditions, this strain produced 244.02 U/mL of cellulase-free xylanase activity, a good production for a wild type. The xylanolytic complex was stable over a wide range pH and the optimal conditions for its activity were 50°C and pH 5.5. These results indicate possible employment of such enzymatic complex in some industrial processes, which require activity in acid pH, wide-ranging pH stability, and absence of cellulase activity. The purification of the main components of this complex and its physicochemical characterization is being development in our lab.

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