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Research Article

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Thermostable Fungal Xylanase as a Supplemental Additive for Commercial Cocktails

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Abstract

Enzymes are crucial in acquiring valuable biotechnological products, such as fermentable sugars, from alternative carbon sources. Lignocellulosic biomass, often derived from agro-industrial wastes, serves as a renewable source for second-generation (2G) ethanol production employing cellulolytic and hemicellulolytic enzymes in the saccharification process, releasing glucose for alcoholic fermentation. Thus, there is a growing demand for novel catalysts that act in biomasses depolymerization, while remaining environmentally friendly. This study aimed to induce xylanase production by Ceratocystis fimbriata using barley, corn cob, and wheat bran as substrates. Additionally, the study evaluated the supplementation of a commercial cellulase cocktail with the partially purified fungal xylanase. The biomass substrates exhibited the highest xylanolytic activity after 8 days of cultivation, with barley yielding 21.20 U/mg. After partial purification through ion exchange chromatography, the xylanase produced in barley demonstrated activity of 67.34 U/mg, resulting in a purification factor of 3.2 and a yield of 56.9 %. The xylanase displayed optimal activity in the pH range of 2.0 to 8.0, retaining more than 50 % of its relative activity. Moreover, the enzyme displayed an optimal temperature of 55 °C and retained over 50 % of its activity after 72 hours at 50 °C. The supplemented Multifect® CL cocktail retained xylanase activity above 50 % for 72 hours, unlike the non-supplemented one. These findings unveiled the thermostability of C. fimbriata xylanase. Finally, the supplementation resulted in hydrolysis enhancement of pretreated sugarcane bagasse, leading to an increase of over 40 and 80 % in glucose and xylose production, respectively.

Keywords: *Ceratocystis fimbriata,* Glycosyl Hydrolases, Thermostability, Biochemical Characterization, Saccharification

1. Introduction

Environmental demands have stimulated several industrial sectors to adapt to the green chemistry principles [1]. Second generation (2G) ethanol consists of utilizing agricultural residues to be hydrolyzed, generating sugars that can be posteriorly fermented into ethanol [2]. The waste utilization produces more economic value from the same initial materials, and contributes to a circular economy, closing the carbon cycle and minimizing impacts [3]. Although 2G ethanol shows a high environmental appeal, the production steps face some challenges. Among the processes involved, enzymatic hydrolysis, also called saccharification, shows a high cost, due to the number of needed enzymes [4]. Xylanase is an essential enzyme that randomly cleaves β-1,4 bonds in the xylan backbone, the most common hemicellulose. Microorganisms are good enzyme producers, mainly for their relatively simple maintenance and fast growing [5]. Fungi secrete many enzymes and can be cultivated in a variety of media, including lignocellulosic biomass. *Ceratocystis fimbriata* is a plant pathogen that infects many agricultural and forest species, and needs to secrete lignocellulolytic enzymes to overcome the plant cell wall barrier [6,7]. This study aimed to use different lignocellulosic biomasses to induce the production of xylanases by C. fimbriata, and to evaluate the supplementation of a commercial cellulase cocktail for increasing cellulose and hemicellulose depolymerization. To the best of our knowledge, this is the first report of a thermostable xylanase from *C. fimbriata.*

2. Materials and Methods

2.1 Culture Conditions for *Ceratocystis fimbriata* LPF1054

The fungus *Ceratocystis fimbriata* LPF1054 was kindly donated by the Forest Pathology Laboratory, Federal University of Viçosa, and it was maintained in PDA (Potato-Dextrose-Agar) plates. Ten mycelium disks were added in semi-solid media with 70% of moisture using barley, corn cob or wheat bran as carbon sources.

The flasks were incubated at 28°C for 12 days and evaluated each 2 days.

2.2 Protein Quantification and Enzymatic Assay of Xylanase

Protein quantification was performed as shown by Bradford [8]. Xylanase assay was estimated by the 3,5-dinitrosalicylic acid (DNS) method [9]. with xylan beechwood 1.25% as substrate and sodium acetate buffer 50 mM, pH 5.0, at 50°C for 15 minutes. The absorbance at 540 nm was used to estimate the xylanase activity, compared to a xylose standard curve. One activity unit (U) was established as the amount of enzyme that releases 1.0 µmol of product per minute in the assay conditions.

2.3 Xylanase Purification

C. fimbriata crude extract was partially purified by Fast Protein Liquid Chromatography (FPLC - ÄKTA pure™), in a Q Sepharose ion exchange column (GE HealthCare) equilibrated with sodium acetate buffer 50 mM, pH 5.0, with a 4 mL/min flow. The salt gradient was performed with the buffer containing NaCl 1.0 M [10]. The fractions with lower protein content and higher enzyme activity were mixed to create a partially purified pool.

2.4 SDS-PAGE

Mini-PROTEAN II System (Bio-Rad) was used to run the SDS-PAGE analysis, with a 12% (w/v) polyacrylamide separation gel and a 5% (w/v) stacking gel. Fifteen µg of protein were injected into the system. The gel was stained with AgNO3 (Blum et al., 1987).

2.5 Enzyme Properties

The optimum pH was evaluated using the McIlwaine buffer from the 2.2-8 pH range [11]. To analyze pH stability, the enzyme was incubated in different pH values for 1 hour. The best activity value was established as 100%. For optimum temperature analysis, the enzymatic assay was performed at 35, 45, 50, 55, 65, and 75°C. For thermal stability, the enzyme was incubated at 50°C for 96 hours. To evaluate its behavior when added to a commercial enzyme cocktail, the thermostability of the Multifect CL® cocktail, the Multifect® XL and the Multifect® CL cocktail supplemented with partially purified xylanase were also investigated. The best activity was considered 100%. The half-life was estimated by using the Curve Expert program.

2.6 Enzymatic Hydrolysis of Pretreated Sugarcane Bagasse

The hydrolysis was performed on alkaline pretreated sugarcane bagasse [12]. The assay was carried out according to da Luz Morales. It aimed to compare the hydrolysis performance of the commercial cocktail Multifect® CL, 10 FPU/g of biomass, and its supplementation with 10 U of xylanase from *C. fimbriata* per g of biomass. The total hydrolysis time was 72 hours, with 1 mL samples taken at 12, 24, 36, 48, and 72 hours. The samples were centrifuged for 15 minutes at 4ºC and 14,500 × g and the supernatant were stored at -20°C.

2.7 Analysis of Enzymatic Hydrolysis Products

Samples were filtered through 0.45 µm filters and glucose and xylose were analyzed by High-Performance Liquid Chromatography – HPLC (CBM-20A/20Alite – Shimadzu) [13].

2.8 Statistical Analysis

The statistical analysis was conducted with ANOVA and Tukey's Test (p-value < 0.05) with the assistance of Minitab® 19.1 software. All experiments were performed in independent triplicates.

3. Results and Discussion

The xylanase activities of *C. fimbriata* after cultivation on barley, wheat bran, and corn cob for 12 days are shown in Table 1. Eight days of cultivation with barley proved to be the optimal time and biomass for inducing *C. fimbriata* xylanase expression. According to Hassan, the arabinoxylan content in barley endosperm cell walls ranged from 5.3-9.0 µg/g (w/w), being the second most abundant polysaccharide in its composition, which may have induced higher xylanase production [14]. Due to the high values of enzymatic activity and considering the low total protein content for subsequent purification, barley was chosen as the inducing biomass to produce xylanase by *C. fimbriata.* After purification, the presence of a single peak of xylanase activity was observed, that was eluted before the salt gradient and contained the activities of positively charged xylanases at pH 5.0 (data not shown). These tubes were collected and polled for enzymatic characterization and saccharification tests. The partially purified pool exhibited xylanase activity of 67.34 ± 1.389 U/mg. This purification was efficient in separating *C. fimbriata* xylanase from other proteins, leading to a purification factor of 3.2 and a yield of 56.9%. These values were confirmed by electrophoresis analysis, which showed a reduced number of protein bands in SDS-PAGE as expected for the partially purified enzyme (data not shown).

Growth time (days)	Barley			Wheat bran			Corn cob		
	Activity (U/mL)	Proteins (mg/mL)	Specific activity (U/mg)	Activity (U/mL)	Proteins (mg/mL)	Specific activity (U/mg)	Activity (U/mL)	Proteins (mg/mL)	Specific activity (U/mg)
2	0.45±0.11	0.08±0.04	4.90±0.25C	0.22±0.04	0.21±0.01	1.05±0.06E	0.00±0.00	0.01±0.001	0.00±0.00D
4	0.32±0.06	0.09±0.01	5.32±0.71C	0.46±0.14	0.22±0.001	2.08±0.09D	0.15±0.10	0.09±0.05	0.90±0.31CD
6	0.43±0.06	0.08±0.003	5.78±0.23C	0.93±0.16	0.20±0.02	4.38±0.36B	0.15±0.11	0.05±0.01	2.69±1.19Ab
8	1.09±0.20	0.01±0.01	21.20±1.78Aa	0.97±0.19	0.21±0.01	5.03±0.22Ab	0.17±0.02	0.06±0.02	2.03±0.26AB
10	0.69±0.04	0.05±0.05	14.66±0.86B	0.76±0.02	0.19±0.02	3.72±0.09C	0.15±0.06	0.11±0.01	1.08±0.12BCD
12	1.83±0.40	0.08±0.003	20.49±0.77A	0.40±0.22	0.20±0.02	1.48±0.15E	0.13±0.10	0.05±0.02	2.50±0.77AB

Table 1: Production of xylanase by *C. fimbriata* grown on barley, corncob, and wheat bran for 12 days. Enzymatic assays were performed in triplicate and the results were expressed as main value±standard deviation. The uppercase letters correspond to statistical differences among the best times within the same biomass, while lowercase letters correspond to statistical differences among the biomasses at their respective best times.

The partially purified xylanase from *C. fimbriata* exhibited the highest activity at pH 5, being an acidic enzyme, and it maintained more than 50% of its relative activity at the pH range of 2.0 to 8.0, indicating its effectiveness across a broad pH spectrum (Figure 1).

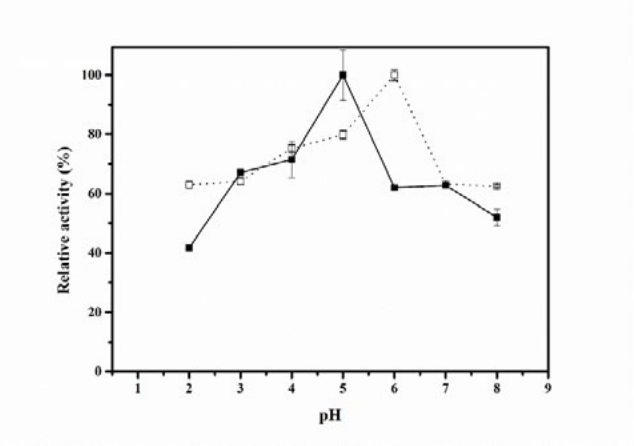

Figure 1: pH effect in the Partially Purified Xylanase from *Ceratocystis fimbriata* Activity (solid line) and pH Stability (dashed line)

At the pH range from 3.0 to 6.0, the enzyme retained 80% of its relative activity, and for the other tested pH values, the enzyme could restore more than 60% of its relative activity, demonstrating high pH tolerance. The optimum temperature for xylanase activity was 55°C. At the temperature range from 50 to 75°C the enzyme activity was more than 50%, indicating a preference for higher temperatures (Figure 2).

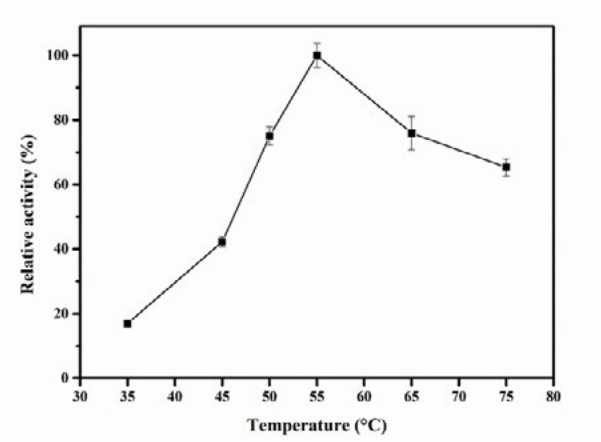

Figure 2: Temperature Effect in the Partially Purified Xylanase from *Ceratocystis fimbriata* Activity.

As shown in Figure 3, the non-supplemented Multifect® CL cocktail lost half of its xylanase activity after 48 hours of incubation, showing t1/2 of 21.54 h.

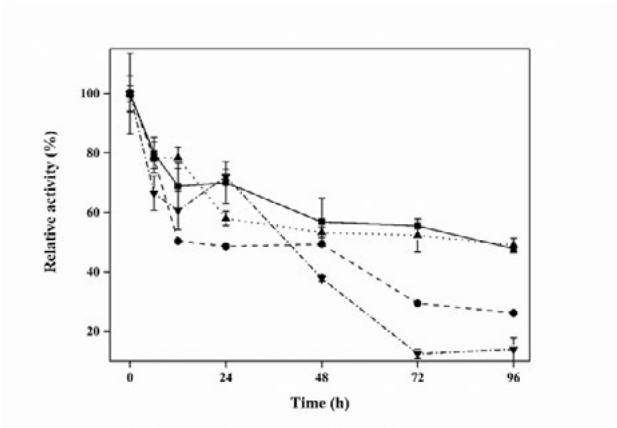

Figure 3: Thermostability at 50°C of the Partially Purified Xylanase from *Ceratocystis fimbriata* (solid line), the Xylanase of the Multifect® CL cocktail (dashed line), the Multifect Cocktail Supplemented with the Partially Purified Xylanase (dotted line) and the Xylanase of the Multifect® XL Cocktail (dash-dotted line).

Multifect® XL, which is composed of hemicelluloses, including xylanases, also exhibited less than 50% of residual activity in 48 hours, with a t1/2 of 29.33 h. However, both the partially purified xylanase from *C. fimbriata* and the Multifect® CL cocktail supplemented with the xylanase exhibited activity values above 50% during 72 h, with a t1/2 of 85.40 and 82.06 h, respectively. These results suggest that although this commercial cocktail has xylanases in its composition, this enzyme is not able to remain active for long periods when incubated at 50°C. On the other hand, when supplemented with the partially purified xylanase from *C. fimbriata*, it showed xylanase activity throughout the 96 hours.

Figure 5 shows the enzymatic hydrolysis profile performed by the commercial cocktail Multifect® CL and its supplementation with the xylanase from *C. fimbriata*. Glucose production was 4.24 g/L, which is equivalent to an increase of approximately 40% in the treatment with the supplemented cocktail when compared to the cocktail without supplementation (Fig. 4A). Therefore, the fungal xylanase was able to increase the ability of the cellulase cocktail in the depolymerization of lignocellulosic biomass. The synergistic action of cellulases and xylanase enzymes is important for greater yields of fermentable sugars [15]. Cellulose is embedded in a tangle of xylan, which to be accessed by cellulases must first be exposed through the action of xylanases that deconstruct the holocellulose structure [16]. As expected, the xylose release was enhanced after supplementation, and the increase was around 80%, representing 3.45 g/L (Fig. 4B). The results suggest that the purified xylanase from *C. fimbriata* shows synergistic potential to act with the xylanases already present in the commercial mixture, as together the release of xylose was much greater than individually. The supplementation is a resource that enables improvements in biotechnological processes, being advantageous to produce cleaner biofuels [17]. The Multifect® CL supplemented with the xylanase from *C. fimbriata* was capable of converting 39.76% of cellulose and 59.60% of hemicellulose in fermentable sugar from the pretreated sugarcane bagasse, while the solely cocktail showed 28.32 and 39.76% of cellulose and hemicellulose rate hydrolysis, respectively.

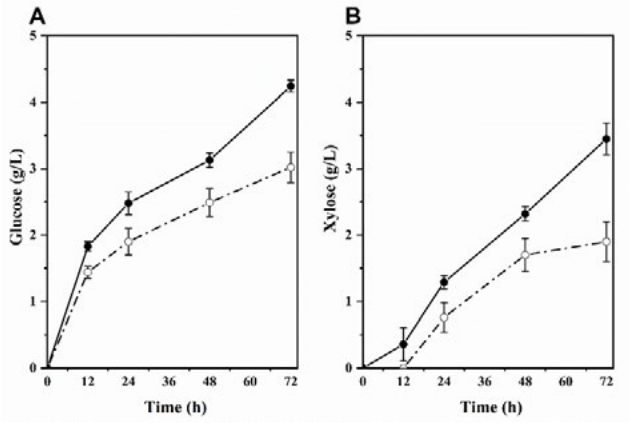

Figure 4: Sugar Release Profile from Enzymatic Hydrolysis of Pretreated Sugarcane bagasse with Multifect® CL Cocktail (Dashed Line) and Multifect® CL with Xylanase Supplementation (Solid Line) for 72 Hours of Reaction. A – Production of glucose. B – Production of Xylose. ab Different Letters Within the Same time Mean Statistical Difference.

4. Conclusion

The *C. fimbriata* xylanase stood out for its capability to enhance the efficiency of a commercially available enzymatic cocktail, acting as a complementary additive to the enzymatic activities present in the mixture. In addition to its complementary role, the partially purified xylanase exhibited superior biochemical characteristics compared to the cocktail, such as higher thermostability [18-22].

Statements and Declarations

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Conflict of Interest Statement

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Author contributions

All authors contributed to the study's conception and design, material preparation, and data collection. Analysis was performed by Carlos Junior de Assis Estevão, Lucas Filipe Almeida, and Gabriela Maitan-Alfenas. The first draft of the manuscript was written by Carlos Junior de Assis Estevão, Lucas Filipe Almeida, Luiz Vinicius de Souza Arruda, João Batista de Souza, and Gabriela Maitan-Alfenas. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability

Data will be available under request.

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