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Biociências

Enzymatic characterization of the lignocellulolytic secretome of Penicillium Variabile wild strain from post-milling sugarcane bagasse in submerged fermentation

Caracterização enzimática do secretoma lignocelulolítico da cepa selvagem de Penicillium variabile proveniente de bagaço de cana-de-açúcar pós-moagem em fermentação submersa

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Abstract: Given its availability of sugars, sugarcane bagasse is a highly favorable environment for colonization by microorganisms, including filamentous fungi that, in the fermentation process, produce efficient enzyme complexes and are among the best evaluated microorganisms for the production of enzymes industrial. In this sense, the present study aimed to evaluate the cellulolytic extract produced by *Penicillium variabile* isolated from post-crushed sugarcane bagasse. The crude enzymatic extract (CEE) was produced by submerged fermentation of the fungus, after partial purification and characterization of the enzymes (fungus supplied gently by BIOMM S/A). The enzymatic activities achieved were 8.772 U/mL of CMCase; 1.975 U/mL β-glucosidase; 0.069 U/mL of FPase; 1.610 U/mL of xylanase and 0.409 mg/mL of total protein (TP). The optimal pH for enzymatic activity of cellulases with *Penicillium variabile* was 4.8, and the stability of these enzymes stood at a pH range of 4.0-4.8. In relation to the thermostability, cellulases remained more stable at -20ºC. The SDS-PAGE profile of the CEE showed proteins in a molecular weight range of 12-100 kDa, and the activities of endoglucanase, β-glucosidase and xylanase were disclosed in the zymogram. The partial purification of CEE by size exclusion chromatography (Sephacryl® S-100) resulted in asymmetric peaks of 60 kDa and 90 kDa, which showed CMCase and β-glucosidase activities, respectively. Thus, our results open horizons for the establishment of biotechnological applications of *Penicillium variabile*, although more studies are needed to understand its physiology and regulatory mechanisms of enzyme production.
Keywords: glycosyl hydrolases, biochemical protein profile, submerged fermentation, enzymatic activities, *Penicillium variabile*.

Resumo: O bagaço de cana-de-açúcar, dada sua disponibilidade de açúcares, é um ambiente altamente favorável à colonização por microrganismos, incluindo fungos filamentosos que, no processo de fermentação, produzem complexos enzimáticos eficientes e estão entre os microrganismos mais bem avaliados para a produção de enzimas industriais. Nesse sentido, o presente estudo teve como objetivo avaliar o extrato celulolítico produzido por *Penicillium variabile* isolado de bagaço de cana-de-açúcar pós-moagem. O extrato enzimático bruto (CEE) foi produzido por fermentação submersa do fungo, após purificação parcial e caracterização das enzimas (fungo fornecido gentilmente pela BIOMM S/A). As atividades enzimáticas alcançadas foram de 8,772 UI/mL de CMCase; 1,975 UI/mL de β-glicosidase; 0,069 UI/mL de FPase; 1,610 UI/mL de xilanase e 0,409 mg/mL de proteína total (PT). Quando verificada a influência de diferentes valores de pH no extrato enzimático em diferentes temperaturas, pode-se observar que as enzimas permaneceram estáveis quando armazenadas a -20ºC e pH 4,0 e 4,8 por até 30 dias. O perfil de SDS-PAGE do CEE mostrou proteínas em uma faixa de peso molecular de 12-100 kDa, e as atividades de endoglucanase, β-glicosidase e xilanase foram reveladas no zimograma. A purificação parcial de CEE por cromatografia de exclusão de tamanho (Sephacryl® S-100) resultou em picos assimétricos de 60 kDa e 90 kDa, que mostraram atividades de CMCase e β-glicosidase, respectivamente. Assim, nossos resultados abrem horizontes para o estabelecimento de aplicações biotecnológicas de *Penicillium variabile*, embora mais estudos sejam necessários para entender sua fisiologia e mecanismos regulatórios de produção de enzimas.
Palavras-chave: glicosil hidrolases, perfil bioquímico de proteínas, fermentação submersa, atividades enzimáticas, *Penicillium variabile*.

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INTRODUCTION

The use of lignocellulosic biomass as raw material for the production of clean and renewable energy consists of a sustainable alternative to the global energy issue, such as second-generation ethanol (SndGE), produced from sugarcane bagasse (Barreto et al., 2025; Uliana et al., 2024; Yaverino-Gutierrez et al., 2024). Plant cell wall that is basically composed of cellulose, hemicellulose, lignin fractions and the lignocellulosic biomass, could be found inby-products/residuesof agriculture, reforestation and sewage solid waste (Piedrahita-Rodríguez et al., 2024). Unlike the use of fossil fuels, the use of biomass for energy generation may offer several advantages, e.g. decreasing of atmospheric carbon dioxide and greenhouse effect by means of the plant's photosynthetic metabolism, which is the propellant engine for biomass production (Zhao et al., 2022).

In the biofuel-making process, the cell wall polymeric chains should be degraded into small units, which results in releasing of monomeric sugars suitable for microbial fermentation (Bordignon et al., 2022). Microorganisms are widely spread in the most varied environments and are involved in the natural enzymatic process of biomass breakdown. Among then, filamentous fungi produce efficient cellulolytic complexes and are among the top-rated microorganisms for producing industrial enzymes (Selim et al., 2025). The genus *Penicillium* has been a potential player in the industrial production of enzymes (Espinoza-Abundis et al., 2023), including cellulolytic enzyme cocktails, as it is known for its ability to hydrolyze substrates such as filter paper, carboxymethylcellulose and cellobiose (Liang et al., 2022; Ogunyewo et al., 2021). In this context, we aim to investigate the hydrolytic profile of a cocktail of partially purified lignocellulolytic enzymes produced by a promising wild strain of *Penicillium variabile* isolated from post-milling sugarcane bagasse.

MATERIALS AND METHODS

All laboratory experiments were carried out at BIOMM Technology S/A, and Unimontes, Minas Gerais, Brazil.

Fungi insolation, inoculum, fermentation and crude enzyme extract

The fungus *Penicillium variabile*, the target of this study, was isolated from sugarcane bagasse after the milling process. *P. variabile* was isolated from the processing of samples in PDA culture medium (20% Potato, 2% Agar, 2% Dextrose), strategically prepared with acidified pH to 5.0 and added with 5 mg/mL tetracycline antibiotic. Sample processing consisted of serial dilution (1/10) from 10g of material from each collection point in sterile saline solution (0.9% NaCl) and then homogenized by shaking for 1 hour. The pH of each sample was then measured and recorded, and aliquots of 100 µL of dilutions 10^{-3}, 10^{-4} and 10^{-5} were plated and incubated at 28ºC ±2 for 7-9 days in PDA. After the growth of the fungi, the individual colonies were counted, plated and differentiated, based on their macroscopic characteristics, in PDA medium, and submitted again to incubation for 7 days at 28ºC ± 2. The microcultivation technique on slides was used for microscopic observation of the reproductive morphological structures typical of filamentous fungi.

The fungal inoculum was prepared in an Erlenmeyer shake flask containing culture medium according to Sreeja-Raju et al. (2020) and using wheat bran as substrate. In order to achieve higher enzyme titers, the fermentation was conducted in a 5 L bioreactor (New Brunswick model Bioflo 3000®) at 30ºC for seven days.

The crude enzyme extract was recovered by filtration of culture broth in a Buchner funnel containing a glass microfiber filter sheet AP20® (Millipore) and filter agent (Supercel® Hyflo, Celite). Then, the crude filtrate was concentrated using a tangential filtration mesh 10 kDa (ultrafiltration) device coupled to a vacuum pump, as in the previous filtration step. The resulting filtrate was evaluated in terms of cellulolytic activities according to the UPAC standard protocol.

Enzyme assays

All enzyme assays were carried out in triplicate and the activities were expressed in International Units, i.e. the amount of a particular enzyme that catalyzes the conversion of 1.0 mmol of substrate per minute (IU/mL). A standard curve was constructed for each different enzyme activity using the enzyme product to be accessed. Carboxymethylcellulase (CMCase) activity was determined according to the methodology described by the International Union of Pure and Applied Chemistry (UPAC) (Ghose, 1987), by measuring reducing sugars released during hydrolysis of carboxymethylcellulose substrate (CMC – Sigma Chemical Co., St. Louis, MO, USA). The reaction medium consisted of 0.250 mL of a 4% (g/L) CMC solution in sodium citrate buffer 50 mM pH 4.8 plus 0.250 mL of enzyme extract. The reaction mixture was incubated at 50°C for 10 minutes and thereafter stopped by the immediate addition of 0.5 mL of DNS (3.5-dinitro salicylic acid), followed by boiling the reaction tubes for 5 minutes. After cooling, samples were read (optical density)/OD) on a spectrophotometer at 540 nm absorbance (Shimadzu UV-1700 UV Pharmaspec Spectrophotometer Shimadzu Pharmaspec) (Miller, 1959).

FPase activity was determined according to standard methods described by the UPAC (Ghose, 1987) based on the measurement of the concentration of reducing sugars released during the degradation of a filter paper tape. The reaction medium consisted of 0.5 mL of the enzyme extract, 1.0 mL of 50 mM sodium citrate pH 4.8 plus a strip of filter paper Whatman No. 1 measuring 1.0 x 6.0 cm (approximately 50 mg). The reaction mixture was incubated at 50°C for 60 minutes and subsequently stopped by adding 0.5 mL of the reaction mixture to a tube containing 0.5 mL of DNS reagent.

β-glucosidase assays were performed in accordance to standard methods described by the UPAC (Ghose, 1987). The method is based on measuring glucose released by the cellobiose hydrolysis reaction. The reaction medium consisted of 0.5 mL of the enzyme extract plus 0.5 mL of substrate solution (15 mM cellobiose in sodium citrate buffer 50 mM pH 4.8). The reaction mixture was incubated at 50°C for 30 minutes and then was stopped by placing the tubes in boiling water for 5 minutes. Glucose was accessed using the analytical kit Glucose GOD-POD® Biosystems.

Xylanase activity was measured using soluble xylan as a substrate (Birchwood Xylan, Sigma Chemical Co., St. Louis, MO, USA). The reaction mixture consisted of 0.1 mL containing the enzyme extract, and 0.2 mL mL 1% xylan solution in 0.05 M acetate buffer pH 5.0. The reaction mixture was incubated at 50°C for 30 minutes and thereafter was stopped by adding 0.3 mL of the solution (DNS) to the reaction tubes for boiling for 10 minutes. The Total Protein (TP) analysis was performed in triplicate, according to Bradford (1976). Then, a non-linear calibration curve was created using Albumin Bovine as standard and samples were read at 590 nm.

SDS-PAGE and zymogram

SDS-PAGE Electrophoresis was accomplished according to the method reported by Laemmli (1970). Protein samples were evaluated in a 10% polyacrylamide gel at room temperature at a constant current of 150 V and 50 mA lasting three to four hours, with the aid of a protein molecular weight (MW) marker ranging from 6-210 kDa Biosciences (Pagemark ™, St. Louis, MO, USA). It was loaded in the gel 10 µg of the MW standard and 50 µg of enzyme concentrate, where the running buffer was a Tris-Glycine buffer pH 8.3.

In order to estimate the molecular weights of fungal enzymes, it was constructed a calibration curve of the relative mobility of proteins composing the MW standard (R) by the logarithm of their molecular weights. Afterward, the fungal proteins MWs were accessed by interpolating between the Rf (retention factor) of a given protein with the calibration curve.

Zymogram analysis was realized using native gel electrophoresis, where 12% polyacrylamide gels were prepared with the addition of specific substrates for each enzyme: xylan (1% solution; w/v) or carboxymethyl cellulose (0.44% solution; w/v). After electrophoretic run, the substrate-added gels were incubated in 40 mM NaOH pH 5.0 buffer for 60 minutes, then soaked in Congo red solution (dye) 0.1%, following by washing gels in 1 M NaCl, which evinced the corresponding bands detected as single halos bands in the gels. To evaluate β-glucosidase activity, a native gel without substrate was incubated, after electrophoresis procedure, in sodium acetate buffer containing 0.3% esculin (Sigma Chemical Co., St. Louis, MO, USA) plus 0.10% chloride ferric for staining until appearing black bands in gel, indicating the enzyme activity.

Size exclusion chromatography

The concentrate enzyme extract was subjected to a size exclusion chromatography (SEC) in a glass column XK®16 / 60 with 12 mL CV (Pharmacia) filled with Sephacryl® S-100 HR (GE) resin, whose stationary phase is formed by porous spheres of polymerized dextran, responsible for the separation of proteins of molecular mass in the range that varies from 1 x 10^3 - 100 x 10^3 daltons. The column was packed by gravity and the eluent used was 50mM sodium acetate buffer plus 100 mM NaCl, pH 4.8. Before loading, the enzyme concentrate was filtered with 0.2 µm filter (Millipore) and then 2.4 mL was loaded in the column. The eluent was continuously pumped into the column by dragging the extract components throughout all column length. Samples corresponding to chromatographic peaks were collected and further analyzed by means of SDS-PAGE and enzyme activities.

Physico-chemical characterization of the enzyme extract

The concentrate enzyme extract was incubated in sodium acetate buffer 50 mM at different pH values (4.0; 4.8 and 6.0) and then stored for 10, 30 and 60 days at 4°C. Aliquots were taken along incubation time for enzyme activities measurements using the same aforementioned substrates in triplicate assays. For the thermal stability assessment, the enzyme preparation was incubated in sodium acetate buffer 50 mM pH 4.8 and stored at -20, 4, 50 and 60°C. All the assays were performed by diluting 3 mL of the sample in 50 mM sodium acetate buffer at a ratio of 1:2 and stored for 10, 30 and 60 days. Samples were collected along incubation time for measuring protein content and enzyme activities using the same aforementioned substrates in triplicate assays.

Statistical analysis

All the experimental data from the enzyme assays and stability studies were analyzed using the software R by means of one-way analysis of variance (ANOVA) and Tukey–Kramer multiple comparisons test.

RESULTS

Enzyme activity profile

The enzymatic profiling of the crude fungal extract from *Penicillium variabile* revealed noteworthy average activities for key lignocellulolytic enzymes. Specifically, carboxymethyl cellulase (CMCase) exhibited the highest activity, with a mean value of 8.772 U/mL, indicating robust endoglucanase function and efficient hydrolysis of amorphous cellulose regions. β-Glucosidase activity reached 1.975 U/mL, underscoring its potential for hydrolyzing cellobiose into glucose and alleviating end-product inhibition during saccharification processes. Xylanase activity, measured at 1.610 U/mL, reflects the fungus's capacity to degrade hemicellulosic components, particularly xylan backbones within plant biomass. Conversely, the filter paperase (FPase) activity, representing the total cellulase complex responsible for crystalline cellulose degradation, was relatively low at 0.069 U/mL, which may suggest a limited exoglucanase or synergistic enzyme action under the conditions tested (Fig. 1).

Figure 1
The average enzyme activity for CMCase, FPase, xylanase and β-glucosidase in the enzyme concentrate of *Penicillium variabile* cultivated under submerged fermentation containing wheat bran as substrate. Values are expressed as triplicate averages

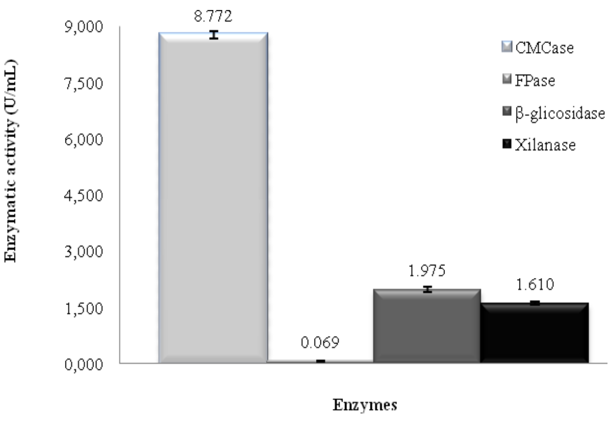
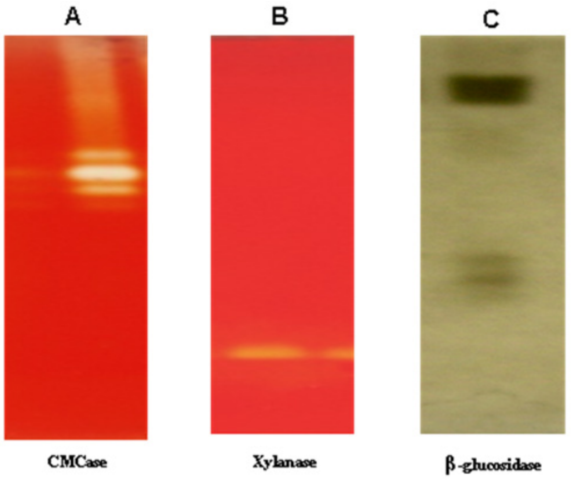


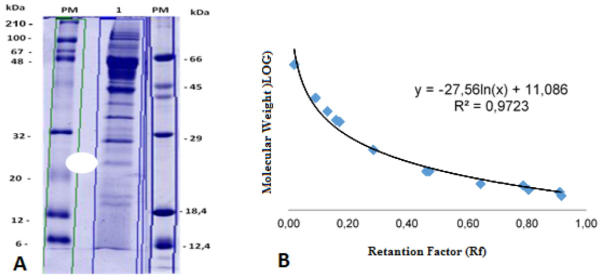
Figure 3
Zymogram of fungal concentrate extract for enzyme activity detection in gels containing specific enzyme substrate: (A) CMCase (CMC as substrate), (B) xylanase (xylan as substrate), and (C) β-glucosidase (esculin as a substrate) activities



CHARACTERIZATION OF FUNGAL SECRETOME USING SDS-PAGE

The secreted enzyme mixture was analyzed by SDS-PAGE, and the gel showed numerous bands corresponding to several proteins with molecular weights ranging approximately from 19 to 119 kDa. (Fig. 2 A and B).

Figure 2
(A) Several proteins present in the enzyme concentrate extract are visible as bands in lane 1 (PM: molecular weight marker). (B) The molecular weights were calculated based on the equation derived from the standard curve obtained by plotting molecular weight (MW) against the relative mobility (Rf) of each protein in the MW standard



The native zymogram gels showed specific bands corresponding to the enzymatic activities of CMCase, xylanase, and β-glucosidase. (Fig. 3).

The chromatogram obtained from size-exclusion chromatography showed eight asymmetric peaks for both the molecular weight (MW) standard and the enzyme concentrate (Fig. 4). Figure 4A shows the chromatogram of the MW standard, with peaks corresponding to bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), myoglobin (18.4 kDa), and HGUT (12.4 kDa) (Sigma).

Figure 4B displays peaks P1 through P8, corresponding to the fungal enzyme concentrate. The estimated molecular weights (MW) of the proteins present in each fraction corresponding to chromatographic peaks P1, P2, P3, P4, P5, P6, P7, and P8 were calculated (Table 1).

Figure 4
Size exclusion chromatography using a Sephacryl® S-100 packed column; (A) Chromatogram of the molecular weight marker and (B) Chromatographic profile of the enzyme concentrate from *Penicillium variabile*

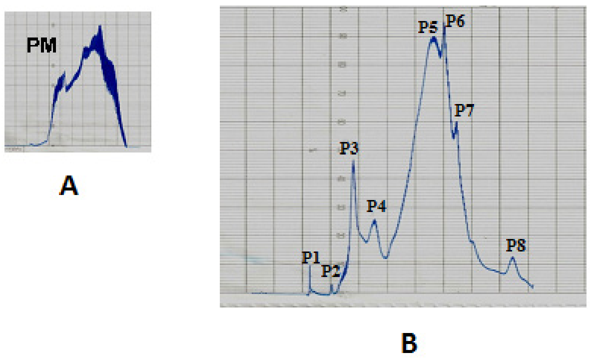


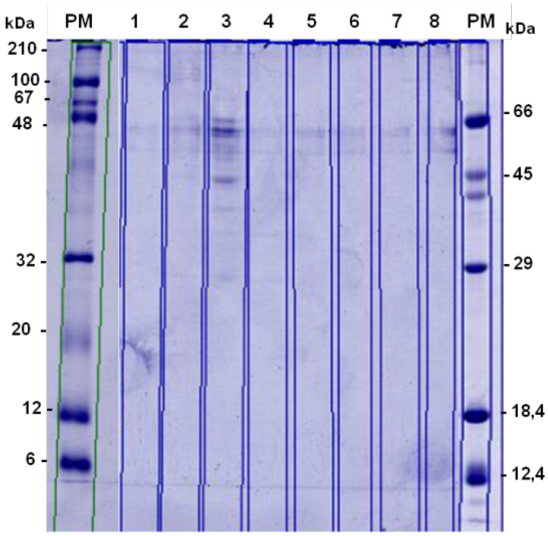
Table 1
Estimated molecular weight (MW) of the proteins present in each fraction of the chromatographic peaks (P1, P2, P3, P4, P5, P6, P7 and P8) from their retention factors (Rf) in the electrophoresis gel

MW (kDa)	Rf	P 1	P 2	P 3	P4	P 5	P 6	P 7	P8
90	0,05	+	+						
89	0,06	+	+						
76	0,09		+						
65	0,14								
61	0,16			+					
60	0,17								
59	0,18						+	+	
58	0,19			+	+	+			+
57	0,21			+	+				
54	0,22								
53	0,23					+	+		+
52	0,26			+	+				
49	0,28								
48	0,31								
46	0,34								
31	0,48								

Note: + means the display of chromatographic peaks detectable by HPLC

Eluted fractions (P1 to P8) were tested for enzyme activities associated with each peak, revealing 0.792 U/mL of β-glucosidase in peak 2 and 4.031 U/mL of CMCase in peak 3, both measured after 240 h of fermentation. FPase and xylanase were not detected in any peak. Lately, the chromatographic fractions were analyzed by SDS-PAGE to estimate the molecular weights of the enzymes responsible for the detected activities (Fig. 5).

Figure 5
SDS-PAGE of fractions eluted from SEC. Lines 1 to 8 correspond to peaks P1, P2, P3, P4, P5, P6, P7, and P8 respectively. PM: molecular weight markers (kDa). Arrows indicate the molecular masses (MM) of the proteins present in peak P3 (lane 3), corresponding to 61, 58, 57, and 52 kDa

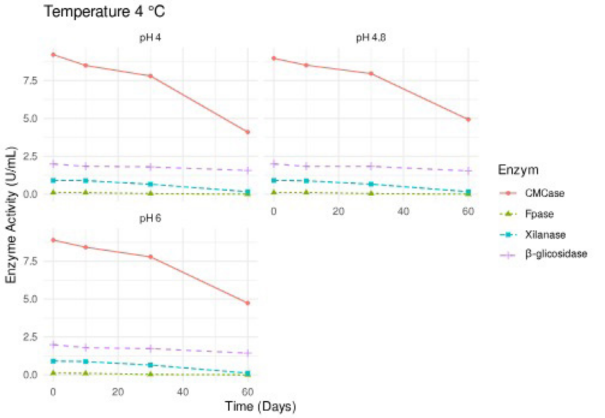


Enzyme concentrate stability

The stability of the tested enzyme activities (CMCase, FPase, β-glucosidase, and xylanase) in the concentrated cell-free fungal extract varied depending on pH and temperature conditions.

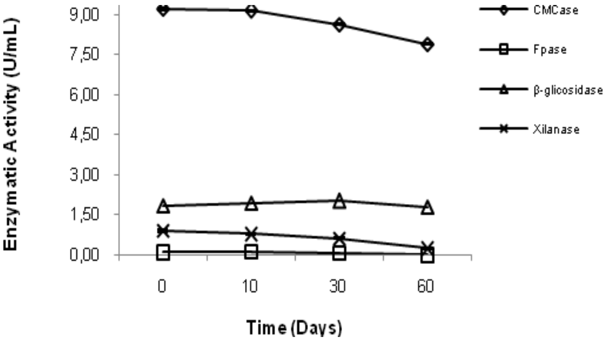
All enzyme preparations showed decreased activity after 60 days of storage at -20°C and pH 4.8. (Fig. 6A, B, and C).

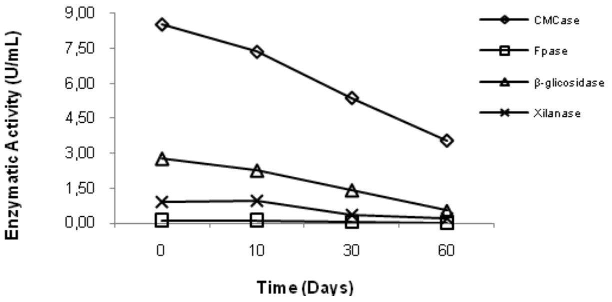
Figure 6
Residual activities of CMCase, FPase, β-glucosidase, and xylanase from *Penicillium variabile* after 10, 30, and 60 days of incubation at 4°C. Enzyme samples were prepared using: (A) sodium acetate buffer at pH 4.0; (B) sodium acetate buffer at pH 4.8; and (C) sodium acetate buffer at pH 6.0

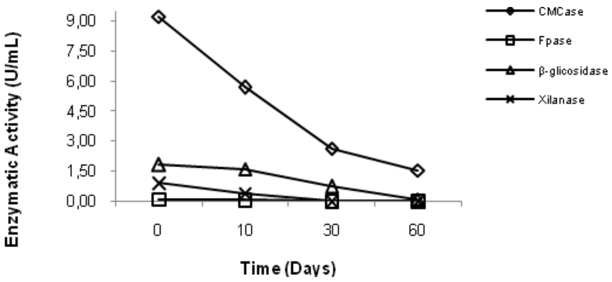


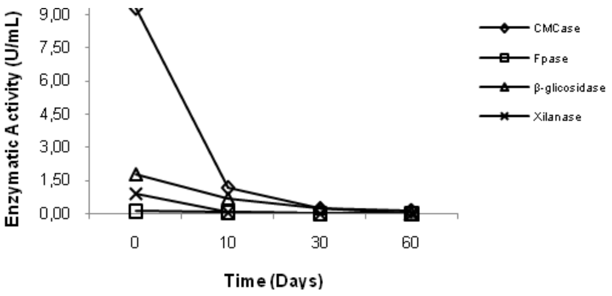
The Figs. 7A, B, C and D show the stability of CMCase, FPase, β-glucosidase and xylanase enzymes when stored in sodUm acetate buffer pH 4.8 at -20, 4, 50, and 60°C for 10, 30, and 60 days. All the enzymes assayed (β-glucosidase, CMCase, xylanase and FPase) had no significant loss in enzymatic activity after 60 days storage at -20°C and pH 4.8.

Figure 7
Assessment of thermal enzyme stability (CMCase, FPase, β-glucosidase, and Xylanase) of Penicillium variabile enzyme concentrate stored at pH 4.8 for 10, 30 and 60 days. The storage temperature was (A) -20°C, (B) 4°C, (C) 50°C, and (D) 60°C









DISCUSSION

Enzymatic activity

Fungi, in general, are known to be good producers of several types of enzymes, the production of which occurs naturally and inherently to the metabolism of each species (Corbu et al., 2023). Sorensen et al. (2013) and Naeem et al. (2022) reported that fungi, such as *Aspergillus*, *Fusarium*, *Penicillium* and *Trichoderma* are natural producers of several β-glucosidases. In this perspective, elucidation of the enzymatic potential of these microorganisms is of great importance, because in addition to enabling the development of enzymatic systems that are not obtainable in plants or animals, it guarantees the supply of enzymes in various segments of production goods and services, such as textile, food, bioenergy industries, etc. In the present study, it can be seen that the wild strain of *Penicillium variabile* has great potential for enzymatic synthesis. This observation is confirmed when we verify the enzyme activity peaks expressed for each enzyme, with 8.772 U/mL in CMCase; 1.975 U/mL in β-glucosidase; 1.610 U/mL in xylanase and 0.069 U/mL in FPase (Fig. 1 in results).

The genus *Penicillium* has a significant number of strains known to be capable of secreting cellulolytic enzymes (Bhat & Bhat, 1997; Long et al., 2023). Insights into the capability of the lignocellulolytic enzymes of Penicillium parvum 4-14 to saccharify corn bran after alkaline hydrogen peroxide pretreatment. Lynd et al. (2002) and Zhao et al. (2023) in your study of novel transcription factor CXRD regulates cellulase and xylanase biosynthesis in *Penicillium oxalicum* under solid state fermentation. *Penicillium* species possess adaptive enzymatic systems capable of responding to lignocellulosic substrates, often through tightly controlled gene expression modulated by carbon source availability, inducer presence, and fermentation conditions. In this sense, *Penicillium* species, such as *P. oxalicum* and *P. janthinellum*, demonstrates how transcriptional regulators (e.g., CXRD) and environmental signals orchestrate the differential expression of enzyme-encoding genes. These regulatory circuits could be similarly active in *P. variabile*, potentially explaining its robust enzymatic output. Ng (2004) and Christopher et al. (2023) show that early cellular events and potential regulators of cellulase induction in *Penicillium janthinellum* NCIM 1366 (Espinoza-Abundis et al., 2023; Goyari et al., 2015). Corroborating with these authors, we verified that the highest value of enzymatic activity obtained in the present study with *P. variabile*, was in the test with CMCase (8.772 U/mL), attesting the enzymatic potential of this fungus in synthesizing enzymes of the cellulolytic complex. This value is significantly lower than those reported in recent studies using similar substrates. For instance, when *Aspergillus niger* ATCC 16888 was cultivated on copra (coconut) waste under submerged conditions, peak CMCase activity reached approximately 3.29 U/mL after 96 hours (Ganeshan et al., 2023). Similarly, a study using sugarcane bagasse and brewery spent grain in submerged culture reported CMCase activity of 3.29 U/mL (Moran-Aguilar et al., 2021). Comparison of submerged and solid-state fermentation for cellulase production by *Aspergillus niger* using coir waste. Bioresource Technology Reports, 14, 100677. In the present study, it was also possible to confirm the ability of *P. variabile* to synthesize the enzyme β-glucosidase, with a peak activity of 1.975 U/mL. From an ecological perspective, the wild strain of *P. variabile* may be adapted to nutrient-variable environments, which could favor the evolution of broad-spectrum enzyme systems. This adaptive trait is beneficial under fermentation conditions using complex carbon sources, such as wheat bran, where multiple enzyme activities must be coordinated. The high CMCase and β-glucosidase levels observed suggest that *P. variabile* can effectively degrade amorphous and crystalline cellulose regions, which is essential for full saccharification. This value was higher than that found by Souza (2011), who found an activity of 1.34 U/mL in submerged culture for the same substrate. In agreement with the findings of this study for the genus *Penicillium*, Ritter et al. (2013) and Silva Lima et al. (2024), the last author uses of an inexpensive carbon source for the production of a cellulase enzyme complex from *Penicillium* ucsense S1M29 and enzymatic hydrolysis optimization using the strain *Penicillium echinulatum* 9A02S1 in submerged cultures containing sorbitol as a substrate, obtained a peak activity of 0.47 U/mL. While Zampieri (2013), in tests with the same species *P. echinulatum* found β-glucosidase peaks of activity of 0.350 U/mL and 0.275 U/mL, in a submerged culture containing cellulose and cellobiose as a substrate, respectively, and for example, in submerged cultures containing cellulose, β-glucosidase levels reached ≈1.15 IU/mL after 120 h, whereas in media containing glycerol or elephant grass, levels were ≈0.27–0.46 IU/mL at 96–120 h. These values are in the same range as the previously reported activities of 0.275–0.350 U/mL (Lenz et al., 2020). These levels contrast with the 0.31U/mL (P47C3, 120h) and 0.10U/mL (P40B3, 96h) reported by Tonelotto et al. (2014), evidencing significant gains in enzyme yield under optimized conditions. The results of this research with *P. variabile* surpass these values even further production lower than that obtained in the present study with *P. variabile* for this same enzyme at different incubation time (For example, *A. niger* HDF05 reached approximately 60U/mL of activity after optimization in submerged culture (wheat bran + $(NH_4)_2SO_4$), a substantially higher value. In submerged fermentation mode with wheat bran and glycerol, another study obtained 9.37IU/mL of β-glucosidase using *A. niger*. The results obtained also point to the potential of *P. variabile* to produce xylanase, having obtained a value of 1.610 U/mL. This value was similar to that obtained by Hoffman and Wood (1985) and Ogunyewo et al. (2020). Engineered *Penicillium funiculosum* produces potent lignocellulolytic enzymes for saccharification of various pretreated biomasses. The study reports xylanase activity of 76 U/mL in submerged fermentation with optimized medium containing wheat bran, who cultivated *Penicillium funiculosum* in submerged fermentation with wheat straw and reported enzymatic activity of 1.87 U/mL for xylanase (Pasari et al., 2023). In agreement with the earlier findings of Poutanen et al. (1987), more recent investigations have demonstrated substantially higher xylanase production by *Aspergillus niger* using wheat bran. Under optimized submerged fermentation conditions with wheat bran, strain AN-13 yielded up to approximately 125 U/mL of xylanase. Likewise, solid-state fermentation using wheat bran by strain BG achieved 4.008 U/g dry substrate, indicating the remarkable enzyme production capacity of this system link (Azzouz et al., 2022). These values greatly exceed the 12 U/mL, reflecting advances in strain optimization and fermentation process control. Submitting the mutant strain of *A. niger* in submerged fermentation containing wheat bran, reported that its activity reached a significant 12.00 U/mL (Ali et al., 2024). Okafor et al. (2007) reported that the maximum enzyme activity peak for xylanase in *A. niger* in submerged cultures containing bagasse as a substrate reached 0.95 U/mL in 96 hours. However, when the substrate was replaced by sawdust or xylan, the enzymatic activities resulted in peaks of 0.65 and 0.80 U/mL in 120 hours, respectively. Recent study have demonstrated that these levels can be significantly surpassed through the use of optimized strains and conditions. In solid-state cultivation, *Aspergillus niger* produced up to 3.18 U/mL of xylanase using alkali-pretreated corn straw. In submerged fermentation, *A. niger* yielded 1.35 U/mL with white sawdust, and 1.25 U/mL and 1.22 U/mL with red and black sawdust, respectively (Fasiku et al., 2023). These observations, in addition to corroborating the potential of the genus *Penicillium* as a lignocellulolytic enzyme producer, demonstrate the importance of the substrate type used in screening for production. These findings are because the enzymatic production values obtained using bran or wheat straw as an inducing substrate, were considerably higher when compared to studies where different substrates were used, such as sugarcane bagasse, sawdust or xylan.

The enzyme that showed less activity was FPase, producing 0.069 U/mL of activity. A similar result was reported by Tonelotto (2012) and Adetunji **and Olaniran (2023).** These authors described an increase in the production of this enzyme, with *A. niger* being 0.13 U/mL in 48 hours for submerged cultures containing wheat bran. Poutanen et al. (1987) reported FPase activity of 3.1 U/mL for the mutant strain *A. awamori* VTT-D-75028 in submerged fermentation with wheat bran. More recent studies confirm that filamentous fungi, including *Penicillium* and *Aspergillus awamori*, can reach or exceed this level with appropriate optimizations. For example, *Penicillium* sp. AKB-24 achieved 1.31FPU/g (~1.31U/g) of FPase in solid culture with wheat bran. Furthermore, *Trichoderma virens* produced about 49.3U/g of FPase in SSF using wheat bran (Badhan et al., 2015; Kumar et al., 2016**).** A similar amount of activity has been described by Singh et al. (2009) evaluating *Aspergillus heteromorphous* under the same conditions of cultivation and substrate. Similar FPase activity levels were reported for *A. heteromorphus* under SSF conditions. Most recently Singh et al. (2021) compared submerged and solid-state fermentations with rice straw, obtaining 6.4 IU/g of FPase in SSF after five days, while in submerged culture they recorded 3.8 IU/g. These values are significantly higher than the 0.13–0.31 U/mL already observed by Tonelotto (2012) in *A. niger* and other classical studies, highlighting the superior potential of SSF with lignocellulosic substrates. Doppelbauer et al. (1987) reported FPase activity of 1.1 U/mL for the mutant strain *T. reesei* MCG-77 in submerged fermentation with wheat straw. Recent studies have shown significant increases in this activity using optimized conditions and pretreated substrates: In a medium containing furfural residues and wheat straw pretreated with H_2O_2, *T. reesei* reached 8.0FPU/mL after ~160h, with a maximum of 8.4FPU/mL at 142h. The mutant strain RC-23-1 showed 6.2FPU/mL in SSF with “avicel”, and up to 4.1FPU/mL in glycolytic medium, demonstrating high efficacy under liquid conditions. These results demonstrate considerable advances in enzyme yield when compared to the initial value of 1.1U/mL for FPase activity. This state-of-the-art result clearly shows how solid-state fermentation (SSF) methods and suitable lignocellulosic substrates can significantly improve FPase production by filamentous fungi of different species and isolation origins (Liang et al., 2022; Zhao et al., 2022). Hoffman and Wood (1985) reported FPase activity of 0.31 U/mL for *P. funiculosum* grown in submerged fermentation with wheat straw. Recent data show significant improvements in optimized strains. For example, *P. funiculosum* ATCC11797 achieved up to 0.354 U/mL FPase after optimization of medium and fermentation conditions. Furthermore, catabolite derepressed mutants, such as strain MRJ-16, produced an impressive 6.47 FPU/mL of filter paper activity in medium optimized for saccharification of plant residues. These results demonstrate that, with genetic engineering or strain selection and optimization of bioprocess conditions, it is possible to significantly exceed the initial values of 0.31U/mL, reaching high levels of enzymatic activity, both in laboratory tests and in industrial application (Castro et al., 2010). A value similar to that obtained to date in these conditions evaluated for *P. variabile*. When comparing the values of enzyme activity described in the literature with those presented in this study, we observed that *P. variabile* demonstrated enzyme activity superior to that presented by reference species in enzymatic production, in the production of CMCase and β-glucosidase enzymes. Thus, we can infer that the wild strain of this species has great enzymatic potential, and further studies are needed to understand not only its physiology, but also the regulatory mechanisms of enzyme production and indution. In addition, the examples presented point to the difficulty in comparing the values obtained with those already described in the literature, because in addition to the experimental variables, such as variations in incubation time and moisture, fungal mutations, etc. There are notable differences in cultivation conditions, type of substrate used, type of fermentation and calculation of enzymatic activity, information that is not always detailed in such publications. Goldbeck et al. (2013) demonstrated, through Pareto graphic analysis only the concentrations had a statistically significant influence and other different parameters did not on the production of cellulolytic enzymes. Subsequent studies confirm and expand this conclusion. For example, in *Acremonium strictum*, it was observed that molasses levels of approximately 2 g/L had a strong negative impact on CMCase and β-glucosidase activities. Similarly, in *Trichoderma reesei*, sugarcane molasses was used as a carbon source in a low-cost medium, contributing to a robust cellular enzymatic pattern, as revealed by secretome analysis (He et al., 2014). Rational engineering of the *Trichoderma reesei* RUT-C30 strain into an industrially relevant platform for cellulase production. In this study, the authors used sugarcane molasses as a carbon source in fed culture, achieving up to 46.8 g/L of extracellular protein in 168 h and demonstrating industrial potential for second-generation ethanol (Xiang et al., 2021). This paper demonstrates significant advances in strain engineering and molasses cultivation strategies, highlighting a significant increase in enzyme production compared to those reported by He et al. (2014) and Fonseca et al. (2020). In summary, *P. variabile* shows strong potential as a source of lignocellulolytic enzymes. Its performance, even under non-optimized conditions, reflects promising biochemical pathways and regulatory networks. Future studies should aim to elucidate these mechanisms through transcriptomic, proteomic, and metabolic analyses, advancing our understanding of fungal enzyme production and enabling its industrial application.

Determination of enzymes' molecular weight

The SDS-PAGE analysis of the enzymatic concentrate from *Penicillium variabile* revealed the presence of protein bands with molecular weights ranging from approximately 12 to 100 kDa (Fig. 2). This broad molecular range indicates a diverse enzymatic profile, characteristic of complex lignocellulolytic systems. These findings are biochemically consistent with prior studies on fungal cellulases. For instance, Tong et al. (1980) identified cellulases and β-glucosidase from *Thermoascus aurantiacus* with molecular weights of 78, 48, 33, and 89 kDa in cultures containing filter paper. More recent research has reinforced this diversity. Dave et al. (2015) and Carvalho et al. (2010) purified endoglucanases from *T. aurantiacus*, reporting molecular masses between 35 and 75 kDa. Proteomic studies confirm the expression of multiple CAZymes with complementary functions, reflecting the evolutionary adaptation of this thermophilic fungus to lignocellulose degradation (Gabriel et al., 2021). Similarly, *Penicillium echinulatum* 9A02S1 exhibited complex protein profiles with bands around 215 and 75 kDa, regardless of carbon source, as reported by Zampieri et al. (2013). Additional work by Schneider et al. (2016) detected xylanase-associated proteins near 240 kDa and other active enzymes at 60–80 kDa via zymography. These molecular signatures are not merely analytical observations, but reflect the physiological response of fungi to complex substrates like wheat bran or sorbitol. The secretion of multiple isoforms and high-molecular-weight enzymes indicates an ecological strategy of functional redundancy and enzyme synergy, critical in competitive environments such as decomposing plant matter, with similar results reported by Todero Ritter et al. (2013). In recent years, *Penicillium echinulatum* variants have emerged as potent cellulase producers and are increasingly recognized as promising candidates for applications in the bioethanol industry (Schneider et al., 2016). These studies confirm the consistent presence of high-weight protein bands (≥ 200kDa) and smaller isoforms (~75kDa), reinforcing the original observations of Zampieri et al. (2013) and expanding the characterization with modern genetic and proteomic data. The pioneering work of Selby and Maitland (1965) already pointed to a wide distribution of enzyme molecular weights in species like *Trichoderma viride* and *Fusarium solani*, spanning 5 to 75 kDa. These findings were later echoed by Khandke et al. (1989), who isolated β-glucosidase (98 kDa), exoglucanase (58 kDa), and endoglucanase (32.2 kDa) from *T. aurantiacus*. The strain-specific and condition-specific expression of enzymes reflects underlying regulatory mechanisms, including transcriptional control and post-translational modifications, which are influenced by factors such as substrate complexity and nutrient availability (Zeng et al., 2016). In the same way that Khandke et al. (1989), purified a β-glucosidase with 98 kDa, exoglucanase with 58 kDa, and endoglucanase with 32.2 kDa produced in submerged cultivation for *Thermoascus aurantiacus* over paper blotter. Subsequently, Grujić et al. (2019) demonstrated that screening *Trichoderma guizhouense* strains on untreated wheat straw enables the selection of isolates capable of efficiently competing for complex natural substrates, leading to the identification of strains that produce enzymes with superior qualitative and quantitative characteristics suitable for industrial applications.

In another study, Takashima et al. (1996, 1999) purifying and characterizing various endoglucanases (24, 32, 42, 45, 46.5 and 58 kDa), six β-glucosidases (46–115 kDa) and a cellobiohydrolase of 38.5 kDa from the supernatant of *Humicola grisea* cultures in submerged fermentation with wheat bran, cellulose and Avicel. Recent proteomic and genomic profiling studies reinforce the diversity and presence of these enzymes. Transcriptomic and genomic analysis of *H. grisea* var. *thermoidea* revealed an extensive repertoire of CAZy enzymes, with several endoglucanases and exoglucanases expressed under different pH conditions, especially in cultures with sugarcane bagasse, with molecular masses compatible with classical profiles. Several studies have purified and characterized highly thermostable β-glucosidases (~50kDa) as well as cellobiohydrolases (CBH1.2, ~49.6kDa) from *H. grisea*, confirming the presence of these enzymatic components through genome and transcriptome analyses, the genetic repertoire of the biotechnological relevant thermophile fungus *Humicola grisea*. Comparative genomics helped us to further understand the biology and biotechnological potential of *H. grisea*. The results demonstrate that this fungus possesses an arsenal of carbohydrate-active (CAZy) enzymes to degrade the lignocellulosic biomass whose genomic and transcriptomic analysis revealed a vast repertoire of CAZy enzymes in *H. grisea*, optimized for lignocellulose degradation under varying environmental conditions (Steindorff et al., 2021). In all these examples, even those that show differences in values by purification methods, studied microorganisms are consistent with the molecular weights of the enzymes produced in the conditions of these bioassays by *Penicillium variabile* in this study. These recent studies corroborate the multi-enzyme protein profile described by Takashima et al. (1999) using modern molecular biology and proteomic techniques to deepen the characterization of these enzymatic systems. These findings collectively suggest that the molecular weight profile observed for *P. variabile* is not only consistent with other filamentous fungi but is also indicative of a robust enzymatic system shaped by evolutionary pressures to efficiently access carbon from complex plant polymers. The combination of biochemical evidence (enzyme molecular mass), ecological inference (substrate response), and recent advances in omics techniques supports the hypothesis that *P. variabile* possesses a sophisticated enzymatic machinery well-suited for industrial bioprocesses, particularly in the biofuel sector. Future studies focusing on transcriptional regulation, enzyme kinetics, and synergistic action under different fermentation strategies would further elucidate the physiological versatility and commercial potential of this strain.

Partial enzymes purification

In this study, enzymatic dosage for xylanase was similar to the β-glucosidase, and it is expected a similar result in the chromatographic profile. However, xylanase revealed no protein peak in the chromatogram and a possible explanation would be that the xylanase has low molecular weight being impossible to detect on the same chromatographic procedure used in this study (Sephacryl S-100). A 20kDa xylanase was purified from *Bacillus pumilus* SSP-34 using ion exchange columns and size exclusion chromatography, where only specific steps revealed the protein, highlighting the need for adjustment of techniques when working with small isoforms. In the same way, a low molecular weight (~19kDa) thermostable xylanase purified from *Aspergillus fumigatus* was identified by ion exchange chromatography without being detected by gel chromatography, indicating limited detection thresholds of these columns (Paramjeet et al., 2021; Wong et al., 1988). The technology applied to enzymatic studies has led some industries to invest in purification strategies of enzymes. In commercial use, purity is a factor of stability, and few are the production and purification protocols of enzymes that achieve results with high efficiency. Most records involve a series of steps that always aim to obtain maximum yield and purity of the protein of interest (Tan et al., 2015; Tonelotto, 2012). The integration of advanced purification strategies in enzymatic bioprocessing has driven industries to adopt multi-step approaches aimed at enhancing enzyme purity, stability, and yield. While achieving high purity often necessitates a complex downstream workflow, these strategies – ranging from chromatographic separation to aqueous two-phase systems – have proven effective in maximizing enzyme performance for commercial applications. These references illustrate how modern purification methods – in particular ATPS, combined chromatography and immobilization – are decisive for achieving high efficiency in industrial processes, improving not only yield and purity, but also stability and economic viability. The integration of ATPS with other separation techniques, such as chromatography or additional phase modification strategies, can further refine purity and yield, making this approach highly versatile for industrial applications (Bekavac et al., 2024; Paramjeet et al., 2021). From a biochemical and industrial perspective, enzyme purification is driven by the trade-off between purity, yield, stability, and cost. Multi-step protocols – including size exclusion, ion exchange chromatography, and aqueous two-phase systems (ATPS) – help optimize this balance. These approaches improve enzyme recovery and activity retention, which is critical for scaling in commercial enzyme production. For instance, hybrid strategies that combine ATPS with polishing steps, continuous-flow systems, and immobilization techniques have demonstrated enhanced yield and economic feasibility in recent studies, including chromatography and continuous flow systems. These references illustrate how modern purification methods in particular ATPS, are crucial for achieving high efficiency in industrial processes, improving not only yield and purity, but also stability and economic viability (Guajardo & Schrebler, 2024; Padhan et al., 2023; Sharma et al., 2025). This phenomenon parallels previous observations, such as a 20kDa xylanase from *Bacillus pumilus* and a ~19kDa thermostable xylanase from *Aspergillus fumigatus*, which required ion-exchange steps for detection and were not visible on gel exclusion columns – highlighting the need to tailor purification strategies to enzyme size and properties.

Riou et al. (1998) in order to purify and characterize the β-glucosidase produced by *Aspergillus oryzae*, using chromatographic processes such as molecular exclusion, and ion exchange, resulting in a chromatographic profile with two protein peaks. After application of these protein fractions on SDS-PAGE, a single band was revealed with a molecular weight of 43kDa. They demonstrated that this enzyme can be present with a variety of molecular weights and, in this particular case, with one of the smallest known molecular weights of aerobic fungi, these weights can range from 39-480kDa for β-glucosidase enzyme. Thongpoo et al. (2014) solated and compared GH3 β-glucosidase enzymes with high glucose tolerance from *Aspergillus niger* ASKU28, using chromatography purification techniques and deep enzymatic characterization. The highly efficient P1.2 β-glucosidase performed better than the commercial β-glucosidase preparation in cellulose saccharification, suggesting its potential applications in the cellulosic ethanol industry. Rojas et al. (1995) with respect to the variety of molecular weights revealed by β-glucosidase enzyme, which according to these authors can be explained by the division of this enzyme into three groups: those that hydrolyze only oligosaccharides, those which exhibit affinity for many types of substrates or those having a higher affinity for the aryl group (organic radical derived from benzene ring) β-glucoside. Godse et al. (2021) in your review article discusses the properties of novel β-glucosidases, including glucose tolerance and activation, substrate specificity, and thermostability, highlighting their potential applications in lignocellulosic biomass degradation, the food sector, and pharmaceutical processes. These enzymes, in contrast to those derived from conventional sources, exhibit characteristics that make them promising candidates for advancements in white biotechnology. The stability of the enzymatic activities evaluated (CMCase, FPase, β-glucosidase, and xylanase) in the concentrated cell-free fungal extract of *P. variabile* was influenced by both pH and temperature conditions. All enzyme preparations exhibited a progressive decline in stability throughout the incubation period, with higher pH levels further exacerbating enzymatic inactivation. The complete decline in enzyme activity across pH and temperature gradients suggests kinetic fragility under non-optimal conditions, necessitating stabilization via purification additives or immobilization – strategies well-documented in enzyme engineering literature. Siqueira (2010) in this partial purification of *Penicillium corylophilum* extracts showed a relevant chromatographic profile only on the Sephacryl S-200 column, revealing a single protein peak associated with xylanase activity. Like show in the results section, size exclusion chromatography using a Sephacryl® S-100 packed column produces chromatographic profile of the enzyme concentrate from *Penicillium variabile*, with estimated molecular weight (MW) of the proteins present in each fraction of the chromatographic peaks from their retention factors (Rf) in the electrophoresis gel that ranged from 90 to 31 kDa in overall size, based on the protein markers used. Tailored downstream design – perhaps utilizing affinity-based or sequential chromatographic approaches – would capture the full spectrum of cellulolytic and hemicellulolytic enzymes, crucial for industrially robust enzyme cocktails.

Physico-chemical characterization of the enzymatic extract

The optimal pH for enzymatic activity of cellulases with *Penicillium variabile* was 4.8, and enzyme stability was best in the pH range of 4.0-5.0. In relation to the thermostability, cellulases remained more stable at -20ºC. According to Steiner et al. (1994) the stability of endoglucanases produced by *Penicillium purpurogenum* was best at pH 4.8, a result similar to that obtained in this research. According Zavaleta and Eyzaguirre (2016) *Penicillium purpurogenum* produces the enzyme PpGAL1, an endo-β-1,4-galactanase expressed in *Pichia pastoris*, with high stability at pH 4–4.5 and 40°C, making it suitable for industrial use. These studies reinforce the optimized pH range between 4.4 and 5.6 for cellulolytic enzymes from *P. purpurogenum*, corroborating the initial data of this work for *P. variabile*, expanding knowledge about enzymatic stability under the broadest possible biotechnological and production conditions, depending on the requirements of each microorganism used. This behavior mirrors that of other acidic-active fungi *P. purpurogenum*, for instance, produces enzymes that are stable, confirming a physiological preference shaped by evolutionary adaptation to moderately acidic niches (e.g., decomposing biomass where organic acids accumulate).

According to Sá-Pereira et al. (2003) the key to success in the protein characterization process is the selection of a technique that considers the stability of the enzymes at different temperatures and pH values. Ozaki and Ito (1991) report that the majority of cellulolytic fungi have better hydrolytic activity in acid pH. Studies conducted by these authors, evaluating the endoglucanase activity produced by *Bacillus* sp. allowed better observation of the enzymatic activity between pH 4.2 to 6.9. Prasanna et al. (2016) working with *Penicillium* sp. showed highest endoglucanase activity after 7 days on Czapek-Dox medium with 0.5% cellulose. Optimal cellulase production occurred at pH 5.0, 30°C, using 0.5% cellulose, lactose, sawdust, and 0.2% yeast extract, yielding 8.7, 25, and 9.52 U/ml of FPase, CMCase, and β-glucosidase, respectively. The stability had become broader, maintaining 100% activity in the pH range of 3.2 to 9.5. The results produced by *P. variabile* show the stability of CMCase, FPase, β-glucosidase and xylanase enzymes when stored in sodium acetate buffer pH 4.8 at -20, 4, 50, and 60°C for 10, 30, and 60 days. All the enzymes assayed (β-glucosidase, CMCase, xylanase and FPase) had no significant loss in enzymatic activity after 60 days storage at -20°C and pH 4.8.

Steiner et al. (1994) with the fungi enzimatic analysis and Fouda et al. (2023), that characterize thermo-tolerant cellulase enzyme produced by *Bacillus amyloliquefaciens* M7, an insight into synthesis, optimization and characterization with the purified cellulase that showed peak activity (73.6 ± 1.1 U/mL) at 50°C, with reduced activity at other temperatures. Similarly, *B. licheniformis* C55 cellulase also peaked at 50°C. That way these different authors reported that the optimum temperature for the enzymatic activity assays of cellulase in a variety of organisms in general is around 50 to 60°C, while the optimum temperature to maintain the stability of the cellulase was -20°C. Temperatures compatible with those obtained in enzyme activity assays and stability of the cellulases produced by *Penicillium variabile* in this study were 50°C and -20°C respectively. Aguiar and Lucena (2011), to evaluate the activity of the enzyme complex produced by *Aspergillus niger* over time (43 days) observed that the enzyme activity remained almost constant when the sample was stored at -18°C. In the same study, however, at a temperature of 4°C, there was a reduction of 43% in their enzymatics activity after 24 hours. Qadir et al. (2018) and Sohail et al. (2009) show enzyme production by solid-state fermentation was also investigated and found to be promising. Highest production of cellulase was noted at pH 4.0, but at 35°C under submerged conditions, differently that was done in this work. Growth and enzyme production was affected by variations in temperature and pH. In this refrigeration (4°C), free water remains available for the development of the organism, justifying the possibilities the degradation of the sample and some enzymatic activities (Szymońska & Wodnicka, 2005), and Moura et al. (2016) when comparing the two types of storage (25°C and -18°C), there was difference only for the activity of galactosidase and trypsin at 60 days. The enzymes of the enzyme complex SSF studied remain stable during the processing of pelleted diet at 55°C, maintaining activity for at least 60 days when stored at temperatures up to 25°C. Alekseyeva et al. (2022) reported that at 4–5°C, there was a reduction of up to 50% in enzyme activity in just one week, attributed to enzymatic hydrolysis and microbial proliferation. Thus, species composition and temperature determine the role of saprophytic and saprobiotic fungi in organic matter and especially in lignocellulosic plant biomass under completely different climatic and working conditions, emphasizing the need for the selection of strains adapted to the best biotechnological conditions for enzyme production. Thermostability tests showed that *P. variabile* cellulases were most stable when frozen at −20°C, while the highest catalytic activity occurred near 50°C. These values align with trends observed in other fungal and bacterial enzymes, reflecting a common biochemical architecture optimized for moderate thermophily – possibly involving stabilizing features like glycosylation and disulfide bonds. The study of Mejía et al. (2024) reveals that enzymatic extracts enhance the insecticidal efficacy of conidial biopesticides regardless of fungal species, contributing to the optimization of biological control agents. Then, the enzymatic crude extract from several fungi (ECE) was concentrated and partially characterized. This characterization consisted of measuring the enzymatic activity (lipase, protease and, chitinase) and determining the enzyme stability after storage at temperatures of − 80, − 20 and 4°C. Protease activity dropped to 77.1% ± 2.0 after 30 days at −20°C. At −80°C and 4°C, the largest decreases occurred within the first 7 days, after which activity remained stable through day 30. Unlike the other studies cited above, in the case of maintenance of the crude enzymatic extract, as verified and evaluated for *P. variabile* under these experimental conditions tested, but especially even at freezing temperatures and storage time, different behaviors and activities were observed for each of the enzymes characterized in this study. In short, it was not possible to generalize the choice of a specific physicochemical parameter that could be applied to all the enzymes evaluated for this species of fungus. Notably, there was no universal physicochemical profile across *P. variabile* enzymes – each displayed distinct stability thresholds. This heterogeneity highlights the intrinsic complexity of fungal enzyme systems and suggests that single-condition preservation strategies are insufficient. Instead, tailored approaches – drawing from fermentation science and protein engineering – are necessary. Further research combining transcriptomics, enzyme kinetics, and protein stability profiling will be essential to fully exploit this strain's biotechnological potential.

CONCLUSION

The present study highlights the enzymatic potential of *Penicillium variabile*, particularly in the production of cellulolytic and hemicellulolytic enzymes such as CMCase, β-glucosidase, and xylanase. Among these, β-glucosidase exhibited the highest specific activity, surpassing the levels reported for *Aspergillus awamori*, a model organism widely recognized in the literature for its efficient production of this enzyme (Ginni et al., 2021; Godse et al., 2021). This finding is especially significant considering that the *P. variabile* strain used in this work is wild-type and non-genetically modified, underscoring its intrinsic enzymatic capacity and biotechnological relevance. The ability of *P. variabile* to produce high levels of β-glucosidase complements previous reports that emphasize the genus *Penicillium* as a promising source of lignocellulolytic enzymes with potential applications in biofuel production, food processing and bioremediation (Long et al., 2023; Lynd et al., 2002). These results contribute to expanding the catalog of fungal species with industrial potential and reinforce the importance of biodiversity in prospecting novel enzyme producers.

Moreover, the data obtained support the need for future studies focused on the physiological and regulatory mechanisms involved in enzyme biosynthesis by *P. variabile*. Optimization of key parameters such as incubation temperature, pH, fermentation time, and the enzyme-to-substrate ratio will be essential to enhance productivity under industrially relevant conditions. Such investigations, coupled with omics approaches (transcriptomics, proteomics), could reveal the regulatory networks that control enzyme expression and improve process scalability.

In conclusion, this work represents a valuable contribution to the understanding of enzyme production by *P. variabile*, offering a strong foundation for its potential application in industrial biocatalysis and second-generation bioethanol production.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data

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