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Research article

Production of thermostable β-glucosidase and CMCase by *Penicillium* sp. LMI01 isolated from the Amazon region

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ABSTRACT

Background: Cellulolytic enzymes of microbial origin have great industrial importance because of their wide application in various industrial sectors. Fungi are considered the most efficient producers of these enzymes. Bioprospecting survey to identify fungal sources of biomass-hydrolyzing enzymes from a high-diversity environment is an important approach to discover interesting strains for bioprocess uses. In this study, we evaluated the production of endoglucanase (CMCase) and β-glucosidase, enzymes from the lignocellulolytic complex, produced by a native fungus. *Penicillium* sp. LMI01 was isolated from decaying plant material in the Amazon region, and its performance was compared with that of the standard isolate *Trichoderma reesei* QM9414 under submerged fermentation conditions.
Results: The effectiveness of LMI01 was similar to that of QM9414 in volumetric enzyme activity (U/mL); however, the specific enzyme activity (U/mg) of the former was higher, corresponding to 24.170 U/mg of CMCase and 1.345 U/mg of β-glucosidase. The enzymes produced by LMI01 had the following physicochemical properties: CMCase activity was optimal at pH 4.2 and the β-glucosidase activity was optimal at pH 6.0. Both CMCase and β-glucosidase had an optimum temperature at 60°C and were thermostable between 50 and 60°C. The electrophoretic profile of the proteins secreted by LMI01 indicated that this isolate produced at least two enzymes with CMCase activity, with approximate molecular masses of 50 and 35 kDa, and β-glucosidases with molecular masses between 70 and 100 kDa.
Conclusions: The effectiveness and characteristics of these enzymes indicate that LMI01 can be an alternative for the hydrolysis of lignocellulosic materials and should be tested in commercial formulations.

1. Introduction

Cellulases are enzymes capable of acting on cellulosic materials, promoting hydrolysis. Cellulolytic enzymes in general have been studied since the second world war, when American soldiers were stationed in the South Pacific realized the deterioration of their objects' cotton base, and later, as a result of various studies led by Dr. Reesei, a yeast strain identified as *Trichoderma viride* was isolated and was determined be the responsible agent for degrading cellulose materials [1]. Scientific research in cellulase has increased, and much of it involves prospecting microorganisms and genetic improvement of cellulolytic species for production. Moreover, the characterization of enzyme complexes has a special emphasis because of its industrial potential [2].

According to the nomenclature of enzymes (Enzyme Commission numbers), cellulases are classified under glycoside hydrolases (EC 3.2.1.). Following the nomenclature of the International Union of Biochemistry and Molecular Biology Committee (NC-IUBMB), these enzymes are highly specific biocatalysts and work synergistically to release sugars. Glucose is of greater industrial interest because of its potential for conversion into ethanol [3,4,5]. Cellulases are enzyme complexes that are classified according to the site of hydrolysis of the cellulose fiber. For example, endoglucanase hydrolyzes cellulose in amorphous regions (i.e., accessible regions in the cellulosic fiber); exoglucanase hydrolyzes the reducing end of the fibers; and β-glucosidase completes the cellulose hydrolysis and releases glucose from cellobiose, cellodextrins, and other oligosaccharides [1,3].

Various microorganisms, including fungi and bacteria, produce a complex of cellulolytic enzymes. Fungi are considered the most

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efficient producers of these enzymes. They naturally produce cellulases and other accessory proteins, including oxidoreductases, which are required for the complete saccharification of lignocellulose [6,7]. The first fungal species described that produced cellulase was *Trichoderma reesei*, which is the most well-studied fungus to date. However, other species (particularly filamentous fungi) have been reported as alternative enzyme sources. Among these fungi are the genera *Aspergillus*, *Penicillium*, and *Trichoderma*. Various *Penicillium* spp. isolates have been investigated for their efficiency of cellulase production, similar or superior to that of standard lineages (i.e., *T. reesei*). In addition, the possibility of producing enzymes under aerobic and extracellular conditions and in large quantities makes fungal cellulases the preferred enzymes in the industry [8,9,10,11].

The main industrial sectors using cellulases are the textile, industrial food, detergent, cellulose pulp and paper, agriculture, livestock, and bioconversion sectors. In these industrial processes, enzymes can be exposed to extreme pH and temperature conditions [12]. Acid cellulose is more adequate to degrade feedstock cellulose in the bioconversion industry, where biomass undergoes acid pre-treatment. The ability to work in an acidic pH environment is also a requirement for enzymes used in the textile industry in the finishing step where they act on cellulolytic fibers [13,14]. Moreover, alkaline and halophilic enzymes are mainly used in detergents [15,16]. The widespread use of cellulolytic enzymes and the increased need to replace fossil fuels probably will make cellulases very valuable enzymes. It is true if ethanol, butanol, or any other product derived from fermentation becomes a mainstream fuel [11,17,18].

Enzyme hydrolysis is an essential component in the process of obtaining ethanol from biomass, and its viability is associated with high conversion efficiency at low production costs. High specificity, low inhibition by its hydrolysis products, and high stability are some of the desirable characteristics for an enzyme to be considered commercially viable [2,19]. The interests and prospection for cellulolytic enzymes has been growing steadily. This is true for industrial saccharification processes for ethanol production. The optimization of polysaccharide biomass conversion into fermentable sugars involves the use of enzyme cocktails that allow greater profitability [20]. Within this scenario, the species of filamentous fungus genus *Penicillium* stand out as great producers of cellulases and are targeted for their enzymes, which are part of the efficient enzyme blends [11].

Penicillium citrinum is abundantly found in the soil, being more common in tropical regions. It has been isolated from the roots, stems, and leaves of coffee plants, cereals, and tropical spices [21,22]. Approximately 75% of the Amazon region has low pH soils [23]. Thus, the aim of this study was to investigate the production and physicochemical characteristics of two important cellulolytic enzymes involved in the hydrolysis of cellulose [endoglucanase (CMCase) and β-glucosidase] obtained from *Penicillium* sp. LMI01, which was isolated from decaying plant material in the Amazon region. The properties of CMCase and β-glucosidase produced by the LMI01 isolate were partially characterized, including the optimum pH range, optimum temperature, and thermostability. Furthermore, the proteins secreted by this fungal species were characterized, which allowed the estimation of the molecular masses of these two enzymes.

2. Materials and methods

2.1. Isolation and identification of the LMI01 lineage

The filamentous fungus *Penicillium* sp. LMI01 was isolated from decaying plant material in the soil from Presidente Figueiredo, Amazonas, Brazil (latitude 01°96′04″S and longitude 60°14′37″W). The fungus was identified morphologically by using traditional methods [24,25,26]. The isolate was preserved following the method proposed by Castellani [27] and reactivated in potato dextrose agar (PDA). For molecular identification, isolate LMI01 was cultured in Czapek-Dox liquid medium, and total DNA was extracted using a Fungi/Yeast Genomic DNA Isolation Kit (NorgenBiotek Corporation, Canada) according to the manufacturer's instructions.

The internal transcribed spacer (ITS) region was amplified by polymerase chain reaction (PCR) using the ITS1 [28] and UniR primers [29]. The reaction mixture included 0.2 mM of each dNTP, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.5 μM of each primer, and 1 U of Taq polymerase (Promega) in a final volume of 25 μL. The amplicons were purified using a Wizard® SV Gel and PCR Clean-up System Kit (Promega) and quantitated in a NanoDrop® (Thermo Scientific). rDNA was sequenced using a BigDye® Terminator V.3.1 Cycle Sequencing Kit (Life Technologies) according to the manufacturer's protocol. The sequences were assembled using SeqManPro™v(DNAStar®). The sequences were obtained (DNAStar), and the consensus contig was deposited in the National Center for Biotechnology Information (NCBI) under GenBank accession no. KU686951. The contigs were searched in NCBI GenBank (https://www.ncbi.nlm.nih.gov/) from the CBS Fungal Biodiversity Centre (http://www.westerdijkinstitute.nl/), which is a database of homologous sequences of closely related species.

2.2. Cellulase production under submerged fermentation

For cellulase production, 1 mL of spore suspension (6.0×10^6 spores/mL) were used to inoculate 500 mL of Mandels and Weber medium [30], modified as described by Szijàrtó et al. [31]., in 2-L conical flasks. The fermentation medium was composed of $(NH_4)_2SO_4$ (5.6 g/L), KH_2PO_4 (4.0 g/L), $CaCl_2.2H_2O$ (0.8 g/L), $MgSO_4.7H_2O$ (0.6 g/L), peptone (1.8 g/L), yeast extract (0.5 g/L), $FeSO_4.7H_2O$ (10.0 mg/L), $MnSO_4.4H_2O$ (3.2 mg/L), $ZnSO_4.7H_2O$ (2.8 mg/L), and $CoCl_2.6H_2O$ (40.0 mg/L) and contained 7.5 g/L of carboxymethyl cellulose (CMC) as the carbon source. The flasks were incubated in triplicate at 30°C for 216 h under constant stirring at 150 rpm. At 24-h intervals, 10-mL aliquots were collected and immediately centrifuged (Centrifuge 5810 R, Eppendorf) for 5 min at 3220 × *g* and 4°C to remove CMC residues and cells. The supernatant (crude enzyme extract) was stored at 4°C until subsequent analysis. Because *T. reesei* is commonly used for the production of cellulolytic enzymes [7,32,33,34], *T. reesei* isolate QM9414 (CCT 2768), which was obtained from the Tropical Culture Collection of the André Tosselo Foundation (Campinas, São Paulo, Brazil), was used as the control for enzyme production. *T. reesei* QM9414 was cultured under the same conditions as *Penicillium* sp. LMI01.

2.3. Quantification of enzyme activity and total proteins

Enzyme activity was quantitated using the standard method for cellulase established by the International Union of Pure and Applied Chemistry (IUPAC) [35]. The CMC substrate was used for the quantification of endoglucanase (CMCase) activity, and the cellobiose substrate was used for the quantification of β-glucosidase activity. Both substrates were previously diluted in 50-mM citrate buffer (pH 4.8). At least five dilutions were used in each assay, and the enzyme reactions were carried out at 50°C for 30 min. Critical dilutions were determined by plotting two dilutions that yielded an amount of glucose higher and lower than the absolute amount of glucose (0.5 mg for CMCase activity and 1 mg for β-glucosidase activity) to graphically represent the amount of glucose released (mg/mL) as a function of the concentration of the diluted crude enzyme extract [35,36]. The enzyme activity was measured as activity units (U), where one unit of activity was defined as the amount of enzyme capable of producing 1 μmol of reducing sugar per min [35]. The total protein content of the crude enzyme extract was quantitated using the Bradford method [37], and the protein concentration was determined using a linear equation previously obtained using a standard solution of bovine serum albumin. To evaluate total cellulase activity, samples were collected from 72 to 216 h (high activity of CMCase and β-glucosidase). Samples were

pooled and submitted to FPase assay (based on filter paper unit, FPU) according to Ghose [35] (Whatman filter No. 1, 1.0 × 6.0 cm, ~50 mg). All assays were performed in triplicate.

2.4. Effect of pH, temperature, and thermal stability

The pH range for the optimum activity of CMCase and β-glucosidase from *Penicillium* sp. LMI01 was determined by measuring the enzyme activity in the crude extract diluted in citrate–phosphate buffer (1:1) [38] prepared at different pH values (2.6, 3.0, 3.6, 4.2, 4.6, 5.0, 5.4, 6.0, 7.0, and 8.0). The effect of temperature on the enzyme activity was assessed by performing enzyme assays at different temperatures (20–80°C) at 10°C intervals using the crude enzyme extract diluted in citrate–phosphate buffer in the optimum pH range established previously. The thermal stability of the enzyme activity was assessed by pre-incubating the crude enzyme extract for 60 min at the temperature that yielded the optimum activity; aliquots were collected every 15 min and assayed with their respective substrates using time zero as the control activity. The residual activity was expressed as a percentage of the control activity.

2.5. Precipitation with ammonium sulfate and fractionation of the crude *Penicillium sp. LMI01* extract

The crude enzyme extracts with higher enzyme activity were pooled and precipitated using ammonium sulfate in a saturation range from 0 to 80% [39] and solubilized in 2.5 mL of 50-mM citrate buffer (pH 4.8). A 0.5-mL volume of the resuspended material was injected into a Superdex 75 10/300 GL gel filtration column (volume 1.5 mL) coupled to an AKTA Purifier System (GE Healthcare). Elution was performed at a flow rate of 0.5 mL/min, and 1.0-mL fractions were collected. Citrate buffer (50 mM, pH 4.8) was used to equilibrate the column and elute the fractions. The total protein of each eluted fraction was quantitated using the Bradford method, and the CMCase and β-glucosidase activities were assayed in the fractions in which proteins were detected (μg/mL). Fractions with enzyme activity were further analyzed.

2.6. Electrophoresis and zymography of CMCase

The crude extract fractions, fractions obtained by precipitation with ammonium sulfate, and fractions obtained by gel filtration were first concentrated 10× using a SpeedVac™ vacuum concentrator. Subsequently, 10 μL of each fraction was mixed with 5 μL of 5× loading buffer (without β-mercaptoethanol) and incubated at 100°C for 5 min. The proteins were separated on SDS-PAGE gel and an activity gel containing 1.5% CMC (zymogram). The molecular weight standard used was the Spectra™ Multicolor Broad Range Protein Ladder (Thermo Scientific), which contained pre-stained proteins in the size range of 10–260 kDa. Both electrophoreses were performed in Tris-glycine buffer at 50 mA and 200 W [40] for approximately 90 min. After electrophoresis, the SDS-PAGE gel was stained with Coomassie Brilliant Blue R-250 and then destained overnight using a distaining solution containing 50% methanol and 12% acetic acid for visualization of the protein bands. The zymography gel was incubated in 1% Triton X-100 buffer (v/v) under gentle stirring for 1 h to remove the SDS, washed three times with distilled water, incubated in reaction buffer (50 mM sodium citrate, pH 4.8) for 2 h, stained with Congo red (0.1%) under agitation for 20 min, and successively washed with 1-M NaCl solution until the bands were visible [41,42,43].

2.7. Statistical analysis

Data were analyzed using BioEstat® version 5.3 software (Mamirauá Sustainable Development Institute, Amazonas, Brazil — http://www.mamiraua.org.br) and plotted using the ORIGIN© version 2016 tool. One-way analysis of variance (ANOVA) was used to determine the optimal period of enzyme production, and a *t*-test was used to compare enzyme activity between *Penicillium* sp. LMI01 and *T. reesei* QM9414. One-way ANOVA with Tukey's test was used to determine the optimum pH, and two-way ANOVA was used to determine the optimum temperature at each pH. The level of significance adopted was 5%. Descriptive statistics were determined for all sample replicates to calculate the mean and standard deviation.

3. Results and discussion

3.1. Morphological and molecular identification of LMI01

The LMI01 isolate was evaluated for 14 d on PDA. This culture grew rapidly and presented radially grooved colonies with white mycelia in the peripheral area, a grayish-green center, and a yellow reverse colony color. The morphology was analyzed using a microculture slide and visualized under a Nikon Eclipse Ni Light microscope (Nikon, Japan). This isolate showed smooth-walled spherical conidia, ampulliform phialides, and divergent metulae that formed interspersed metulae of uniform size. These characteristics allowed the isolate LMI01 to be classified as belonging to the genus *Penicillium*. The ITS sequence of LMI01 deposited in NCBI under GenBank accession No. KU686951 showed 100% similarity with the sequence from *Penicillium citrinum* isolate NRRL 1841 (GenBank accession No. AF033422.1). The genus *Penicillium* comprises a group of fungi that are commonly found in soil as decomposers of various types of organic plant materials [44]. The species *P. citrinum* is the most common in tropical soils and is less frequent in temperate soils [22]. Several species of *Penicillium* are efficient producers of cellulases [9,10,45]. *Penicillium* sp. LMI01 is a new isolate, and therefore, a promising candidate for the optimization of the production of enzymes used in biocatalysis-dependent processes.

3.2. Quantification of the total soluble protein in the crude extract

The total soluble protein content (mg/mL) of *Penicillium* sp. LMI01 was lower than that of *T. reesei* QM9414 during all periods of submerged fermentation (Fig. 1). The highest protein concentration obtained was 0.057 mg/mL for *T. reesei* QM9414 and 0.044 mg/mL for *Penicillium* sp. LMI01 in 168 h ($P = 0.003$). *Trichoderma* spp. are known to naturally produce large amounts of extracellular proteins [46] compared with *Penicillium* spp. The protein concentration in a commercial enzyme preparation of *T. reesei* (60.04 mg/mL) was much higher than the protein concentration found in the concentrated enzyme extract of *Penicillium echinulatum* (0.149 mg/mL) [34]. Similarly, the protein content of *Trichoderma harzianum* (96.4 mg/mL) was much higher than that of *Penicillium funiculosum* (2.6 mg/mL) [47].

In contrast, *Penicillium* spp. have high cellulase activity and low concentrations of other extracellular proteins when cultured on cellulose [48]. Additionally, higher protein levels in *Penicillium* spp. often occur during later periods of submerged fermentation, which coincides with reduction in the mycelial mass and/or increased β-glucosidase activity. This finding suggests that the low abundance or absent proteins in the first hours of fermentation are derived from mycelial degradation and extracellular secretion of β-glucosidase [49]. In the present study, the protein content (mg/mL) in the crude enzyme extract of *Penicillium* sp. LMI01 increased during the same period in which the highest β-glucosidase activity was observed (i.e., between 168 and 192 h of submerged fermentation).

3.3. Volumetric and specific enzyme activity

Penicillium sp. LMI01 showed a peak in the volumetric endoglucanase (CMCase) activity at 72 h (0.60 U/mL). This result was similar to that observed for *T. reesei* QM9414 but with a slightly higher activity value

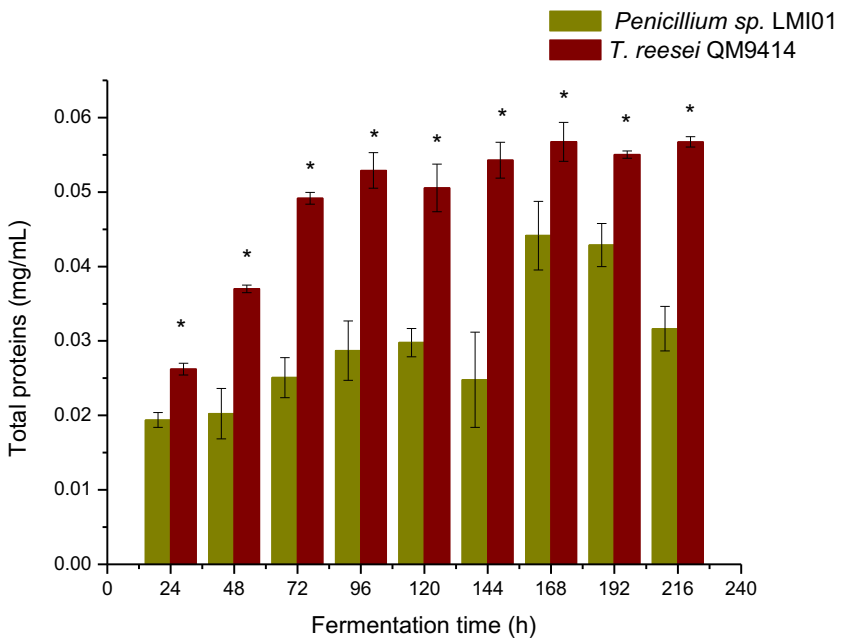

Fig. 1. Total protein profile in the culture supernatant (crude extract) of *Penicillium* sp. LMI01 under submerged fermentation for 216 h. The values represent the means and standard deviations (N = 3). (*) Significant differences in the protein concentrations (mg/mL) between isolate LMI01 and *T. reesei* QM9414 ($P < 0.05$).

(0.86 U/mL). For both species, the volumetric enzyme activity decreased at 96 h. After 120 to 216 h of fermentation, there was no significant difference in the volumetric CMCase activity between the two species (Student's *t*-test, $P > 0.05$), whereas the enzyme activities were significantly different at 48, 72, and 96 h ($P = 0.0017$, $P = 0.0529$, and $P = 0.0093$, respectively). The result demonstrates that volumetric enzymatic activity (U/mL) of *Penicillium* sp. LMI01 was lower than commercial standard *T. reesei* QM9414 activity (Table 1 and Table 2). The peak of volumetric CMCase activity at 72 h was followed by lower activity at 96 h of submerged fermentation, which confirmed the result observed for *Penicillium echinulatum* 9A02S1 in CMC medium [48]. Moreover, CMCase activity (U/mL) in *Penicillium* sp. was previously detected after 72 h of fermentation in liquid medium containing sugarcane bagasse as the cellulosic material [50].

The specific enzyme activity is the volumetric activity (U/mL) in relation to the total protein concentration (mg/mL) and is expressed in U/mg. The kinetic profile of *Penicillium* sp. LMI01 endoglucanase (CMCase) under submerged fermentation for 216 h showed a peak at 72 h, followed by decrease at 96 and 120 h, having another peak at 144 h (Fig. 2a).The specific CMCase activity of *Penicillium* sp. LMI01 was higher than that of *T. reesei* QM9414 (Table 1 and Table 2), corresponding to 24.1 and 24.17 U/mg for *Penicillium* sp. LMI01 and 17.5 and 13.2 U/mg for *T. reesei* QM9414 at 72 h and 144 h, respectively. Volumetric β-glucosidase activity was higher in *Penicillium* sp. LMI01 crude extract than in *T. reesei* QM9414 extract during almost all fermentation periods. At 168 h, the activity of *Penicillium* sp. LMI01 crude enzyme extract was 0.048 U/mL and reached 0.058 U/mL at 216 h, whereas the activity of *T. reesei* QM9414 crude extract was 0.013 and 0.019 U/mL during the same periods ($P = 0.0001$), respectively. The volumetric activity levels in *Penicillium* sp. LMI01 were similar to that of *T. reesei* QM9414 only at 24, 48, and 72 h and reached 0.013 and 0.012 U/mL at 72 h, respectively ($P > 0.05$). In this respect, several studies reported higher β-glucosidase activity during the later fermentation periods of filamentous fungi. For example, *P. echinulatum* activity levels were higher between 96 and 132 h of fermentation [48], whereas the highest activity in *Penicillium* sp. and *Aspergillus sydowii* occurred after 120 h of fermentation [50,51].

The kinetic profile of *Penicillium* sp. LMI01 β-glucosidase increased along the entire submerged fermentation. At 96 h, this activity was 0.817 U/mg in *Penicillium* sp. LMI01 and 0.185 U/mg in *T. reesei*. The specific β-glucosidase activity was higher in *Penicillium* sp. LMI01 crude extract than in *T. reesei* QM9414 extract (Table 1 and Table 2). The highest β-glucosidase activity was observed at 216 h, corresponding to 1.345 and 0.342 U/mg in *Penicillium* sp. LMI01 and *T. reesei* QM9414,

Table 1
Cellulase activity of *Penicillium* sp. LMI01 during periods with higher activity[a].

Enzymatic Activity[b]	Fermentation time (h)[c]	Total proteins (mg/mL)[d]	(U/mL)[d]	(U/mg)[d]
FPase	pooled extracts	0.038 ± 0.002	0.077 ± 0.003	2.031 ± 0.069
CMCase	72	0.025 ± 0.003	0.604 ± 0.044	24.08 ± 1.376
	120	0.032 ± 0.003	0.597 ± 0.029	18.87 ± 0.816
	144	0.025 ± 0.004	0.594 ± 0.021	24.17 ± 2.430
	216	0.043 ± 0.003	0.607 ± 0.027	14.09 ± 0.534
β-Glucosidase	96	0.029 ± 0.005	0.023 ± 0.003	0.817 ± 0.081
	192	0.043 ± 0.003	0.054 ± 0.004	1.227 ± 0.078
	216	0.043 ± 0.003	0.058 ± 0.004	1.345 ± 0.099

[a] Periods (hours) of submerged fermentation with higher enzymatic activity.
[b] Enzymatic activity in culture supernatant (crude enzyme extract).
[c] Submerged fermentation time in hours.
[d] Values refer to means and standard deviation (N = 6).

Table 2
Cellulase activity of *T. reesei* QM9414 during periods with higher activity[a].

Enzymatic Activity[b]	Fermentation time (h)[c]	Total proteins (mg/mL)[d]	(U/mL)[d]	(U/mg)[d]
FPase	pooled extracts	0.054 ± 0.003	0.083 ± 0.003	1.530 ± 0.021
CMCase	72	0.049 ± 0.001	0.860 ± 0.021	17.493 ± 1.264
	120	0.051 ± 0.003	0.655 ± 0.006	13.009 ± 0.917
	144	0.054 ± 0.002	0.715 ± 0.024	13.159 ± 0.387
	216	0.057 ± 0.001	0.671 ± 0.037	11.830 ± 1.159
β-Glucosidase	96	0.053 ± 0.002	0.010 ± 0.001	0.185 ± 0.006
	192	0.055 ± 0.001	0.011 ± 0.001	0.190 ± 0.018
	216	0.057 ± 0.001	0.019 ± 0.002	0.342 ± 0.030

[a] Periods (hours) of submerged fermentation with higher enzymatic activity.
[b] Enzymatic activity in culture supernatant (crude enzyme extract).
[c] Submerged fermentation time in hours.
[d] Values refer to means and standard deviation (N = 6).

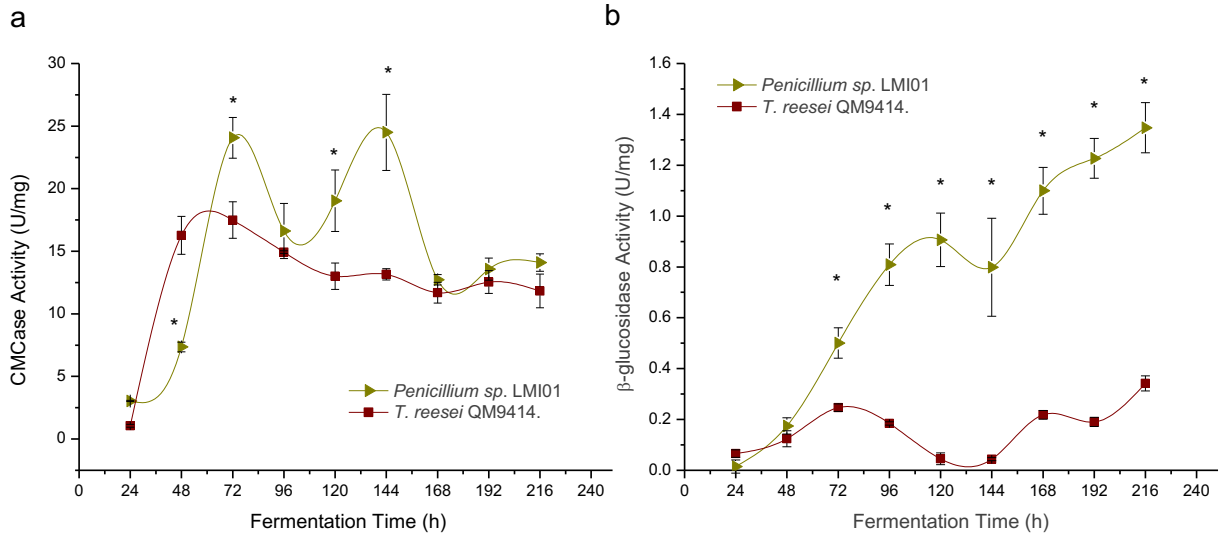

Fig. 2. Kinetic profile of enzyme production (U/mg) by *Penicillium* sp. LMI01. The fungi were cultured under submerged fermentation with CMC as the carbon source for 216 h at 30°C and 150 rpm. Samples were collected regularly at 24-h intervals. (a) CMCase activity, (b) β-glucosidase activity. Values represent the means and standard deviations (n = 3). (*) Significant difference in production between LMI01 and *T. reesei* QM9414.

respectively (Fig. 2b). *T. reesei* is a model fungus because of its efficient cellulase production (primarily endoglucanases and exoglucanases) and is the most studied fungal species. However, its β-glucosidase production is low, corresponding to approximately 1% of the total secreted protein content [52,53]. Several studies have indicated that *Penicillium* fungi are efficient producers of β-glucosidases. In this respect, the β-glucosidase activity of *P. echinulatum* (1.28 U/mg) was higher than the activity found in *T. reesei* (0.29 U/mg) [34]. Additionally, both *P. citrinum* YS40-5 and an isolate of *P. janthinellum* from India produced high levels of β-glucosidase [54,55]. These results together with the present results demonstrate that *Penicillium* sp. LMI01 is a good candidate for the production of β-glucosidases because of its ability to secrete this enzyme at levels equivalent to those reported in efficient cellulolytic fungi.

Total cellulase activity in the extract pooled from *Penicillium* sp. LMI01 was very similar to that of the control *T. reesei* QM9414. FPAse activity was 0.077 FPU/mL for LMI01 and 0.083 FPU/mL for QM9414, which was significantly different (Student's *t*-test, $P > 0.05$) (Table 1 and Table 2). When considering total protein concentration mg/mL, FPase specific activity was higher in LMI01 (2.031 FPU/mg) than in QM9414 (1.530 FPU/mg) (Student's *t*-test, $P = 0.0007$). These results demonstrate that most of the proteins secreted by *Penicillium* sp. LMI01 are cellulolytic complex enzymes. Thus, this microorganism is a promising candidate for optimization for cellulase production.

3.4. Effect of pH on Penicillium sp. LMI01 CMCase and β-glucosidase activity

The CMCase activity of *Penicillium* sp. LMI01 was higher at acidic pH (Fig. 3a). Although the CMCase activity was highest at pH 4.2 (0.409 U/mL), there was no significant difference in the activities at this pH and those at pH 3.6 (0.401 U/mL), 4.6 (0.405 U/mL), and 5.0 (0.395 U/mL) ($P > 0.05$). Similar results were found for the optimum pH for endoglucanase (CMCase) activity of several filamentous fungi, including *P. funiculosum* (pH 4.82), *P. citrinum* (pH 5.5), *Penicillium chrysogenum* (pH 5.0), and *Aspergillus niger* (pH 5.0) [56,57,58,59]. The activity of *Penicillium* sp. LMI01 β-glucosidase was highest at pH 6.0 (0.038 U/mL); however, the activity levels at pH 5.0 (0.032 U/mL), 5.4

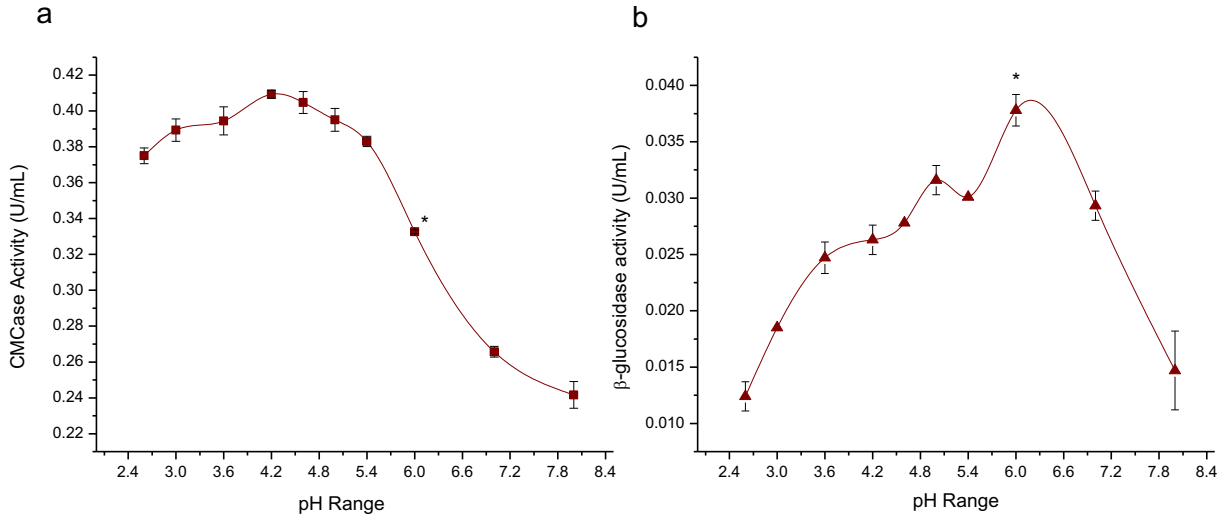

Fig. 3. Effect of pH on *Penicillium* sp. LMI01 enzyme activity. (a) CMCase activity at different pH values, (b) β-glucosidase activity at various pH values. Values represent the means and standard deviations (n = 3), (*) Significant differences at $P < 0.05$.

(0.030 U/mL), and 7.0 (0.029 U/mL) were similar to those obtained at pH 6.0 (Fig. 3b).

In contrast to the observations for CMCase, the β-glucosidase activity of *Penicillium* sp. LMI01 increased at pH values slightly farther from the acidic range. This result confirms the result obtained with *P. citrinum* YS40-5, whose optimum pH for β-glucosidase activity varied between pH 5.0 and 6.0 [54], and *A. sydowii*, whose activity was the highest at pH 6.0 [51]. *Penicillium* sp. LMI01 cellulases are of biotechnological interest because they are active at acidic pH and are generally more desirable for bioconversion (e.g., in conditions in which the biomass needs to undergo acid pre-treatment or needs to be active in combination with *T. reesei* enzymes, whose optimum activity is around pH 5.0 [60], or with *Aspergillus* enzymes, whose optimum pH range is between 4.0 and 6.0).

3.5. Effect of temperature on the activity and thermal stability of Penicillium sp. LMI01 CMCase and β-glucosidase

The optimum temperature for CMCase activity at pH 3.6, 4.2, 4.6, and 5.0 was 60°C ($P < 0.0001$) (Fig. 4a). The optimum temperatures of cellulases from filamentous fungi vary between 30 and 55°C [1]. This result was similar to the result obtained for fungi that were active at temperatures higher than 50°C, including *P. funiculosum*, whose optimum temperature for endoglucanase was 58°C [59], and *P. citrinum* MTCC 6489, whose optimum temperature was 60°C [56]. The optimum temperature for *Penicillium* sp. LMI01 β-glucosidase was also 60°C at pH 5.0, 5.4, 6.0, and 7.0 ($P < 0.0001$) (Fig. 4b). The enzyme activity decreased starting at 70°C for both CMCase and β-glucosidase. The thermal stability of the CMCase and β-glucosidase from *Penicillium* sp. LMI01 was measured at 50, 60, and 70°C. The CMCase activity was stable at 50 and 60°C, with 95.7% of the relative activity (%) maintained after 60 min of pre-incubation compared with the activity of the non-pre-incubated samples (i.e., samples at time zero) (Fig. 4c). For LMI01 sample, incubation at 50 and 60°C had a positive effect on the β-glucosidase activity because the enzyme activity was higher after pre-incubation for 15 min compared with the activity at time zero ($P < 0.01$) (Fig. 4d), and this activity remained constant after pre-incubation for 60 min, with relative activities higher than 100%. The thermostability of β-glucosidase from LMI01 at 50°C was similar to the activity of three recombinant β-glucosidases from *Humicola insolens* Y1; however, at 60°C, this enzyme was more stable than the *H. insolens* Y1 enzyme [61].

Therefore, the thermostability of the cellulases produced by *Penicillium* sp. LMI01 needs to be studied as this property has industrial

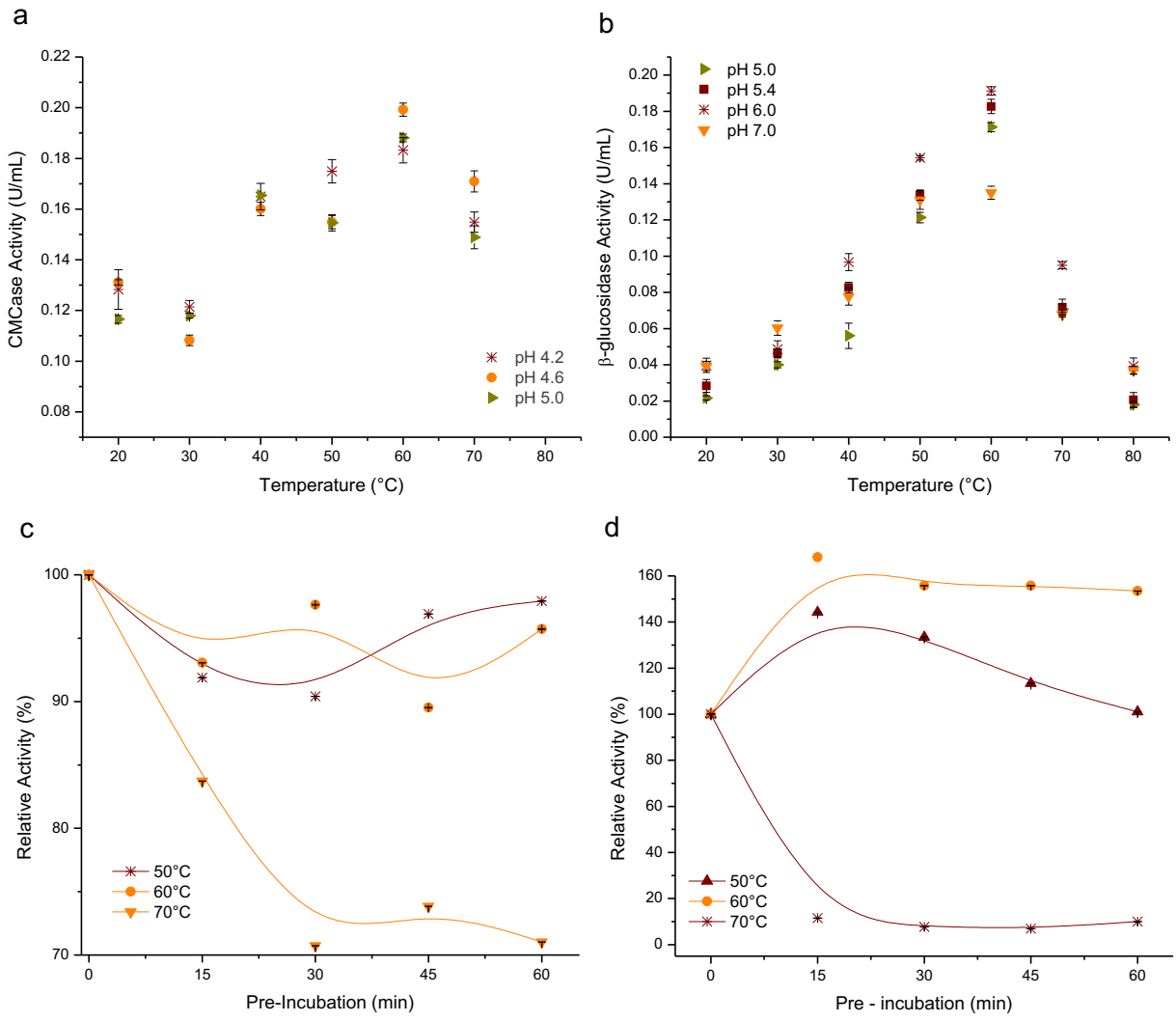

Fig. 4. Effect of temperature and thermal stability on *Penicillium* sp. LMI01 enzyme activity. The optimum temperature was determined at the optimum pH range, and the thermal stability was analyzed in residual activity at temperatures between 50 and 70°C at the optimum pH: (a) Optimum temperature for CMCase activity, (b) optimum temperature for β-glucosidase, (c) thermal stability of CMCase, (d) thermal stability of β-glucosidase. The values represent the means and standard deviations (n = 3). (*): Significant differences at $P < 0.01$ using ANOVA and Tukey's test.

Table 3
Fractionation of proteins from *Penicillium* sp. LMI01 crude extract[a].

Steps	CMCase activity				Protein[b] mg/ml	β-Glucosidase activity			
	U/mL	Yield %	Recovery factor	U/mg		U/mL	Yield %	Recovery factor	U/mg
Crude extract	0.405	100	1	10.69	0.038	0.126	100	1	3.31
Precipitation saturation 80%	0.766	189.0	0.5	5.33	0.144	0.550	438.2	1	3.83
Fractions chromatography									
F 11	ND	ND	ND	ND	0.001	0.046	36.3	15	50.67
F 12	0.071	17.5	38	408.35	0.000	0.026	21.0	46	151.57
F 13	0.181	44.8	4	41.44	0.004	0.017	13.4	1	3.83
F 14	0.103	25.4	4	42.22	0.002	0.010	7.6	1	3.94
F 15	0.056	13.8	6	60.36	0.001	0.007	5.7	2	7.75

ND: not detected, no detectable enzymatic activity.
[a] Crude enzyme extract formed by culture supernatants from collection in periods of high enzymatic activity.
[b] Total protein present in solution, determined by the Bradford method.

importance [62]. In industrial processes, mainly those related to lignocellulolytic biomass conversion to ethanol, enzymatic reactions are carried out at high temperatures, favoring better substrate solubility and consequently better reaction rate; moreover, high temperature also reduces the risk of contamination by undesirable microorganisms [19, 63]. The optimum temperature of LMI01 enzymes was near to the range of some enzymatic complexes used to produce biofuel, including the following commercial blends: Novozymes Cellic®CTec2 (45–50°C) and Accellerase®Trio™ Genecor (40–57°C). In fungi, optimum temperatures for the endoglucanase activity range generally between 50 and 60°C and stability until 50–55°C [64]. An advantage presented by LMI01 is its stability in the range of 50 to 60°C [63].

3.6. Fractionation of proteins in the crude Penicillium sp. LMI01 extract and the CMCase zymogram

Saturation with 80% ammonia sulfate allowed over 100% yield with CMCase, but regarding its purity (specific activity), the purification index was below 1 (one), and specific activity after the purification step was inferior. This shows that this stage probably contributed to the precipitation of other proteins except endoglucanase present in solution. However, this step was most effective in precipitating proteins with β-glucosidase activities, producing an enzymatic activity yield higher than 100% and resulting in a recovery factor of 1.16 fold (Table 3). In the fractions eluted from the gel filtration column, the highest protein concentration was detected in fractions 11 to 15, and enzyme activities were found in these fractions (Fig. 5). CMCase activity was found in fractions 12 (0.071 U/mL), 13 (0.181 U/mL), and 14 (0.103 U/mL); therefore, the CMCase activity of these fractions was analyzed by zymography. CMCase activity was significantly higher in fraction 13 ($P < 0.01$; ANOVA, Tukey's test). β-glucosidase activity was found in fractions 11, 12, 13, 14, and 15; however, the activity in fractions 11 (0.043 U/mL) and 12 (0.025 U/mL) were higher, and fraction 11 presented β-glucosidase activity but not CMCase activity. The gel filtration chromatograph was efficient, presenting specific activity gain and recovery factor above 1 in the fractions with CMCase activity (fractions 12–15). The highest yield occurred in fraction 13, followed by fraction 14; thus, these fractions were chosen for determining the activity in the gel. As shown in Fig. 5, these samples also presented higher concentration of total proteins (μg/mL) and therefore could be visualized on SDS-PAGE. Although we may have lost the degree of purity (CMCase), the yield was sufficient to observe the presence of CMCase and determine its molecular weight by zymography and SDS-PAGE.

Zymographic analysis of CMCase activity revealed a halo corresponding to a molecular mass of approximately 35 kDa in fractions 13 and 14 in the fractionated samples and crude extract (Fig. 6a), which corresponded to the same mass visualized in the Coomassie Brilliant Blue-stained gel (Fig. 6b). However, a band of approximately 50 kDa with CMCase activity was also visualized. This result confirms of the result of a previous study on filamentous fungi that showed that the cellulolytic systems of many fungi produced multiple endoglucanases

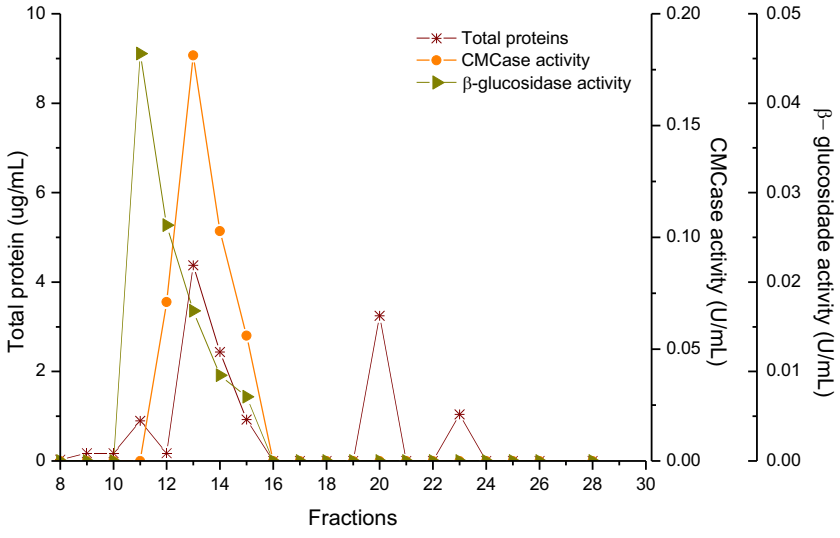

Fig. 5. Enzyme and protein profiles of the eluted fractions. CMCase and β-glucosidase activity (U/mL) and protein content (μg/mL) of the fractions eluted from the Superdex 75 10/300 GL gel filtration column.

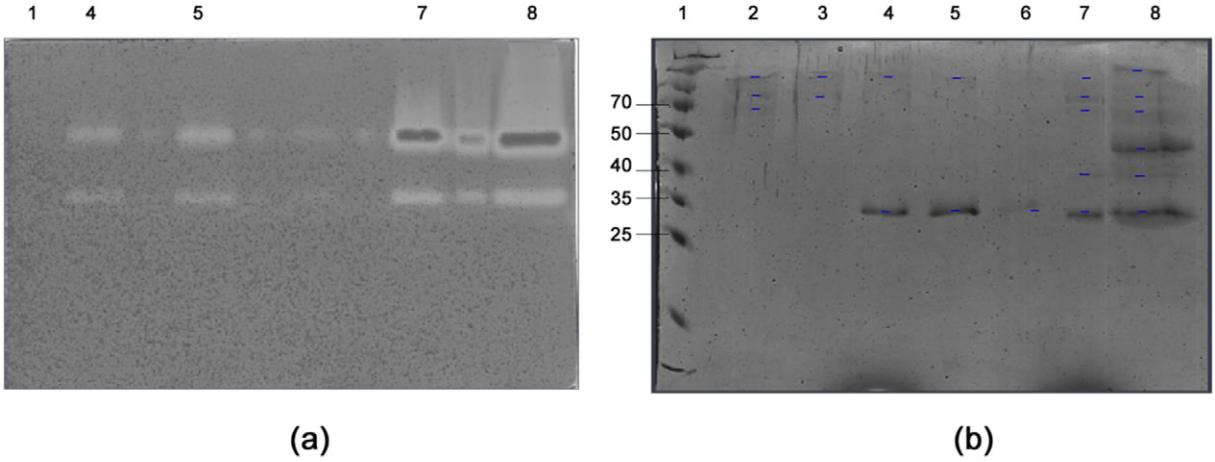

Fig. 6. Zymogram of CMCase and electrophoretic profile of the gel filtration fractions with detectable proteins and enzyme activity. (a) Zymogram of the CMCase activity in SDS-PAGE (1.5% CMC) stained with Congo red, (b) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie Brilliant Blue R-250. 1: standard molecular weight marker (10–260 kDa); 2: Fraction 11; 3: Fraction 12; 4: fraction 13; 5: fraction 14; 6: fraction 15; 7: precipitate (0–80% saturation with ammonium sulfate); 8: vacuum-concentrated crude enzyme extract.

with different molecular masses; generally, the molecular masses of the endoglucanases varied between 32 and 100 kDa [65].

The electrophoretic profile (SDS-PAGE) of CMCase indicated the following protein concentrations (w/v):fraction 11 (0.9 μg/mL), fraction 12 (0.2 μg/mL), fraction 13 (4.4 μg/mL), fraction 14 (2.4 μg/mL), and fraction 15 (0.9 μg/mL). Protein bands with molecular masses of approximately 35 kDa were found in fractions 13 and 14 (Fig. 6b, wells 4 and 5) and fraction 15, although with a lower intensity (Fig. 6b, well 6). Proteins with similar molecular weights were also found in the samples precipitated with ammonium sulfate and in the crude extract (Fig. 6b, wells 7 and 8). The fractionated samples and crude extract also contained protein bands with an apparent molecular mass of approximately 50 kDa. In fractions 11 and 12 (Fig. 6b, wells 2 and 3), the 35- and 50-kDa bands were not visualized. However, fraction 11 contained bands with molecular masses between 70 and 100 kDa. These bands were also present in the fractionated samples, however, with a lower intensity.

The molecular masses of the β-glucosidases produced by microorganisms vary between 45 and 250 kDa [65]. In the present study, the electrophoretic profile of the proteins in fractions 11 and 12 eluted from gel filtration contained proteins with molecular masses between 70 and 100 kDa. Notably, fraction 11 presented β-glucosidase activity but not CMCase activity. Therefore, the molecular masses of the β-glucosidases produced by *Penicillium* sp. LMI01 might be in this range. This finding corroborates the results of previous studies that reported secretion of β-glucosidases with molecular masses of 70 [66,67], 90 [45,66,68,69], and 120 kDa [54] by fungi of the same genus.

4. Conclusion

Our results indicated that the extracellular proteins of *Penicillium* sp. LMI01 were well represented by the enzymes of the cellulolytic complex because the specific activity of CMCase was high compared with that of the commercial isolate *T. reesei* QM9414 under the same culture conditions. Regarding β-glucosidase production, the volumetric (U/mL) and specific activities (U/mg) of *Penicillium* sp. LMI01 were higher than those of *T. reesei* QM9414. This result is promising because β-glucosidase is essential for the increased production of glucose from cellulosic substrates as it acts on oligosaccharides and dimers derived from the initial hydrolysis of cellulases and releases monomers from reducing sugars. This result, combined with the stability of CMCases and β-glucosidases at their optimum temperatures (indicated by the maintenance of high residual activities), demonstrates the potential of *Penicillium* sp. isolate LMI01 for the production and use of cellulolytic enzymes in industrial processes.

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