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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