Genetics and Molecular Research

Identification and characterization of genes related to cellulolytic activity in basidiomycetes

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Genet. Mol. Res. 15 (3): gmr.15038722
Received April, 25, 2016
Accepted June 2, 2016
Published September 16, 2016
DOI http://dx.doi.org/10.4238/gmr.15038722

ABSTRACT. Enzymes produced by basidiomycetes that are involved in the cellulose degradation process, and their respective codifying genes, must be identified to facilitate the development of novel biotechnological strategies and applications in the agro-industry. The objective of this study was to identify prospective cellulase-producing genes and characterize their cellulolytic activity, in order to elucidate the potential biotechnological applications (with respect to vegetal residues) of basidiomycetes. The basidiomycete strains *Lentinula edodes* U8-1, *Lentinus crinitus* U9-1, and *Schizophyllum commune* U6-7 were analyzed in this study. The cellulolytic activities of these fungi were evaluated based on the halo formation in carboxymethyl cellulose culture medium after dyeing with Congo red. The presence of cellulase-codifying genes (*cel*7A, *cel*6B, *cel*3A, and *egl*) in these fungal strains was also evaluated. *L. edodes* and *S. commune*

presented the highest cellulolytic halo to mycelial growth radius ratio, followed by *L. crinitus*. Four genes were amplified in the *L. edodes* strain, whereas three and one genes were isolated from *L. crinitus* and *S. commune*, respectively. The *cel*6B gene (*L. edodes*) presented the conserved domain glyco_hydro_6 and characterized as cellobiohydrolase gene. The results of this study contribute to the existing knowledge on cellulases in basidiomycetes, and serve as a basis for future studies on the expression of these genes and the characterization of the catalytic activity of these enzymes. This allows for better utilization of these fungi in degrading vegetal fibers from agro-industrial residues and in other biotechnological applications.

Key words: Basidiomycetes; Cellulase; *Lentinula edodes*; *Lentinus crinitus*; *Schizophyllum commune*

INTRODUCTION

Lignocellulose is the main component of plant biomass and the most abundant renewable organic resource in the world, with broad applications (at a relatively low cost) in agro-industry. However, the physical and chemical composition of lignocellulose is very complex; this allows lignocellulose to resist chemical and biological attacks, complicating its hydrolytic conversion into materials with a higher aggregated value.

Lignocellulose consists of cellulose, hemicellulose, and lignin. Cellulose is a fibrous, insoluble, and crystalline homopolysaccharide composed of linear chains of D-glucose linked by β-1,4 glycosidic bonds. The strong alignment of its chains and its structural stability confer cellulose with resistance to enzymatic attacks (Bayer et al., 1998). However, several fungi have been shown to promote enzymatic hydrolysis of cellulose through the action of an extracellular cellulolytic complex. Chief among these are basidiomycetes, which produce these enzymes to hydrolyze vegetable fibers.

Cellulolytic fungi produce an enzymatic system composed of three groups of enzymes: endoglucanase or endo-β-1,4 glucanase (E.C. 3.2.1.4), exoglucanase or exo-1,4-β-glucanase (E.C. 3.2.1.91), and β-D-glucosidase (EC 3.2.1.21) (Song et al., 2008). Cellular hydrolysis occurs in oligosaccharides and glucose when there is complete synergy between these enzyme groups.

The system of regulatory genes that codify cellulolytic enzymes, such as cellulase (which is controlled at the transcriptional level), is complex and well-studied (Gutiérrez-Rojas et al., 2015). The various genes coding for cellulase have already been sequenced in basidiomycetes such as *Agaricus bisporus* (Yagüe et al., 1996) and several *Polyporales* (Hori et al., 2013). Novel cellulolytic enzymes and their respective codifying genes in fungi must be identified to understand the multi-enzymatic degradation process, to develop new strategies for the utilization of vegetal residues, and to determine their potential biotechnological applications.

Therefore, the objective of this study was to identify prospective cellulase-producing genes in basidiomycetes and characterize their cellulolytic activity, to elucidate the potential biotechnological applications (with respect to vegetal residues) of basidiomycetes.

MATERIAL AND METHODS

Biological material

Three species of basidiomycetes, obtained from the Culture Collection of the Laboratory of Molecular Biology, Universidade Paranaense, were evaluated in this study: *Lentinula edodes* (Berk.) Pegler U8-1, *Lentinus crinitus* (L.) Fr. U9-1, and *Schizophyllum commune* Fr.U6-7. The strains were cultivated in potato dextrose agar (PDA; 39 g/L) at 28° ± 1°C in the dark; these cultured strains were used to produce the inoculum.

Cellulolytic activity

The cellulolytic activity was determined as described previously by Jo et al. (2011), with some modifications. The mycelium was grown in culture medium containing 10 g/L carboxymethyl cellulose, 5 g/L peptone, 5 g/L yeast extract, 5 g/L K_2HPO_4, 1 g/L NaCl, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.6 g/L $(NH_4)_2SO_4$, and 15 g/L agar. The culture medium, which was autoclaved at 121°C for 20 min, was inoculated with a 5-mm PDA disk containing mycelia, and incubated at 28° ± 1°C for 7 days. Subsequently, 10 mL Congo red solution (2 g/L; prepared with 0.1 mM Tris-HCl buffer at pH 8.0) was added to the mycelium. The dye was discarded after 10 min and the dish was washed twice with a 1 M NaCl solution. The formation of a light-yellow halo in contrast with the red background indicates the production of cellulase. The thickness of the halo and the radial mycelial growth were measured by a pachymeter (± 0.02 mm) and the ratio between the halo thickness and the mycelial radius was calculated.

DNA extraction

The strains were grown in 100 mL liquid medium supplemented with 2% (m/v) malt extract at 28° ± 1°C for 7 days. The mycelial biomass was separated by filtration and washed twice with 30 mL ultrapure water. DNA was extracted using a method described by Raeder and Broda (1985). The mycelial biomass was transferred to a porcelain mortar, macerated with liquid nitrogen, mixed with 2.1 mL/g extraction buffer (1 M Tris-HCl, pH 8.0, 5 M NaCl, 0.5 M EDTA, pH 8.0, and 10% SDS), and incubated at 65°C for 15 min. The resulting lysate was extracted twice, once with phenol and again with a chloroform-isoamyl alcohol (24:1) mixture. After each extraction, the material was centrifuged at 15,200 *g* for 5 min. The extracted DNA was precipitated with isopropanol, recovered by centrifugation (15200 *g* for 5 min), washed twice with ethanol [once with 70% ethanol (v/v) and again with 95% ethanol (v/v)], and centrifuged again (15200 *g* for 15 min). The obtained DNA was re-suspended in ultrapure water and stored at -80°C. The quality and quantity of extracted DNA were verified by electrophoresis on an agarose gel (0.8%; m/v), staining with ethidium bromide, and visualizing the gel with a UV transilluminator. The gel was then photographed by a photo image documenter (L-PIX - Molecular Imaging; Locus Biotecnologia, São Paulo, SP, Brazil). Lambda phage DNA was used as the molecular mass standard to quantify DNA (Life Technologies, Carlsbad, CA, USA).

PCR amplification of cellulase genes

The 50-µL PCR mix was composed of 0.15 mM dNTPs, 1X PCR buffer (20 mM Tris

base, pH 8.4 and 50 mM KCl), 1.5 mM $MgSO_4$, 0.2 µM primers, 2.0 U Taq DNA polymerase, 25 ng template DNA, and ultrapure water. DNA was amplified in a thermocycler (Mastercycler Gradient; Eppendorf, Hamburg, Germany) with an initial denaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 50°-58°C for 1 min, extension at 72°C for 2 min; and a final extension at 72°C for 7 min.

The primers used were specific to endoglucanase and exoglucanase, with annealing in conserved regions of genes from the cellulase enzymatic complex. Primers specific for the *egl* gene of *Agaricus bisporus* (Morales and Thurston, 2003) were utilized for endoglucanases (EC 3.2.1.4): forward primer, 5'-CACCCGTCAGCGGAAGCGAT-3' and reverse primer, 5'-GGGCGCTGGTGCAGGGTAAG-3'. Primers specific for *cel*7A (forward, 5'-GAGCAGCA CCAGGGCCAGTTTTG-3' and reverse, 5'-TCTACGGCCCAGGTCTCACAGTCG-3') and *cel6*B (forward, 5'-GCGTTCCGAATCCAGCACCAA-3' and reverse, 5'-CGGCAACCCCTT CACTGGTTACG-3'), both from *L. edodes* (Lee et al., 2001), and *cel*3AC (forward, 5'-CAACCTGCGCTTCGGGCTCA-3' and reverse, 5'-AGGGCAGGGTTGGCATTCGC-3') (Chow et al., 1994) and *cel*3A (forward, 5'-CAACCTGCGCTTCGGGCTCA-3' and reverse, 5'-AGGGCAGGGTTGGCATTCGC-3') (Yagüe et al., 1996), both from *A. bisporus*, were utilized for exoglucanases (EC 3.2.1.91).

The amplified products were electrophoresed on a 0.8% agarose gel stained with ethidium bromide, using the Ladder 1 kb Plus marker (Life Technologies) as the molecular size standard. The electrophoresed gel was visualized and photographed under UV light by a photo image documentation equipment (Locus Biotecnologia).

Sequencing of PCR products

The amplified products were purified using the Purelink PCR Purification kit (Life Technologies) according to the manufacturer protocols, and sequenced by an ABI-Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer protocols. The nucleotide sequences were submitted to GenBank (NCBI).

Identification of obtained gene sequences and presence of conserved protein domains

The obtained sequences were analyzed for similarity with the gene sequences deposited in the GenBank database, utilizing the BLASTn program (NCBI). Conserved protein domains were identified by analyzing the similarity between our proteins and the protein sequences deposited in the GenBank database, utilizing BLASTx search tool (NCBI).

RESULTS

The results regarding the production of cellulolytic enzymes are summarized in Table 1. The ratio of the degradation halo thickness to the mycelial growth radius indicates the enzymatic activity. Fungi showing a higher ratio of degradation halo thickness to the mycelial growth radius show greater cellulolytic activity per millimeter of mycelium. In this study, this ratio varied from 0.30 to 2.20, with *L. edodes* U8-1 and *S. commune* U6-7 showing the best cellulolytic activity (Table 1).

Table 1. Mycelial growth radius and cellulolytic halo thickness.

Fungus	Strain	Cellulolytic halo thickness (mm)	Mycelial radius (mm)	Ratio between halo thickness and mycelial radius
Lentinula edodes	U8-1	11.75 ± 1.07	5.37 ± 0.47	2.20 ± 0.35
Lentinus crinitus	U9-1	7.17 ± 1.44	23.58 ± 0.22	0.30 ± 0.06
Schizophyllum commune	U6-7	7.51 ± 0.95	5.64 ± 0.67	1.33 ± 0.56

Cellulase genes were amplified from all strains of fungus (Table 2). Four cellulase genes were amplified from *L. edodes* U8-1, whereas three and one genes were amplified from *L. crinitus* U9-1 and *S. commune* U6-7, respectively (Table 2).Two *L. edodes* U8-1 *cel*6B gene sequences were obtained from the amplification products and deposited in GenBank (NCBI) (Table 3). These sequences showed 100% similarity with *L. edodes* gene sequences previously deposited in GenBank. The *cel*6B gene identified in *L. edodes* U8-1 was characterized according to the presence of a conserved protein domain, by analyzing the similarity between the predicted protein sequence and the protein sequences previously deposited in GenBank, using the BLASTx algorithm (Table 4).

Table 2. PCR amplification of basidiomycete cellulase genes.

Gene	Enzyme	Strain	Fungus
*cel*7A	Exoglucanase	U8-1	*Lentinula edodes*
*cel*6B	Exoglucanase	U8-1	*Lentinula edodes*
*cel*3AC	Exoglucanase	U9-1	*Lentinus crinitus*
*cel*3A	Exoglucanase	U6-7	*Schizophyllum commune*
		U8-1	*Lentinula edodes*
		U9-1	*Lentinus crinitus*
egl	Endoglucanase	U8-1	*Lentinula edodes*
		U9-1	*Lentinus crinitus*

Table 3. Identification of gene sequences obtained from PCR amplification of *cel*6B (cellulase), and their similarity to sequences deposited in the GenBank database (NCBI).

Strain	Sequence size (bp)	GenBank accession No.	Description of identified gene, origin species* (GenBank accession No.)	Maximum identity (%)	E-value
Lentinula edodes U8-1	150	KT447633	Cellulase CEL6B (*cel*6B) mRNA, complete cds *Lentinula edodes* (AF411251.1)	100	$2e^{-69}$
Lentinula edodes U8-1	198	KT447633	Cellulase CEL6B (*cel*6B) mRNA, complete cds *Lentinula edodes* (AF411251.1)	100	$2e^{-95}$

*Only those GenBank sequences with a similarity score >90% with the obtained gene sequences were used in the analysis.

Table 4. Identification of conserved protein domains from gene sequences obtained by PCR amplifications of *cel*6B gene (cellulase), and their similarity to amino acid sequences deposited in the GenBank database (NCBI).

Strain	Sequence size (bp)	GenBank accession No.	Description of protein, origin species*, (GenBank accession No.)	Description of identified preserved domain	Maximum identification (%)	E-value
Lentinula edodes U8-1	150	KT447633	Cellobiohydrolase *Lentinula edodes* (AAK28357.1)	Glyco_hydro_6	100	$4e^{-26}$
Lentinula edodes U8-1	198	KT447634	Cellobiohydrolase *Lentinula edodes* (AAK28357.1)	Glyco_hydro_6	100	$5e^{-36}$

*Only those GenBank sequences with a similarity score >80% with the obtained amino acid sequences were used in the analysis.

DISCUSSION

Basidiomycetes have a wide range of biotechnological applications (Umeo et al., 2015); these fungi are known to survive on several types of vegetal material and environments primarily because of their ability to utilize a number of natural carbon sources and degrade cellulose and other polysaccharides (Rytioja et al., 2014). The basidiomycetes strains analyzed in this study were confirmed to produce cellulases (Table 1).

L. edodes U8-1 showed a higher ratio of degradation halo thickness to mycelial growth radius, indicating its higher cellulolytic activity during the degradation of substrate-containing carboxymethyl cellulose. *L. edodes*, a white-rot fungi, produces ligninases that are used in paper bleaching and the biodegradation of xenobiotics. This fungus is also an excellent producer of endoglucanases, exoglucanases, and β-glycosidases. Pereira Júnior et al. (2003), who studied the activity of *L. edodes* cellulases, detected endoglucanase activity in liquid media containing carboxymethyl cellulose and microcrystalline cellulose, showing that the concentration as well as type of cellulose affected the enzymatic activity. Therefore, *L. edodes* has excellent cellulolytic potential, is able to degrade several carbon sources, and presents activities that are related to, and dependent on, the substrate composition.

The white-rot fungus *S. commune* U6-7, the second-highest producer of cellulase in this study (Table 1), can degrade all the components of the vegetal cell wall using a host of hemicellulose- and cellulose-degrading enzymes such as β-glycosidases and exoglucanases (Tsujiyama and Ueno, 2011). Additionally, it is reportedly an excellent lignin degrader (Rytioja et al., 2014). This species is considered as a model for degradation because of its capacity to produce a diverse set of enzymes that can degrade lignocellulosic substrates (Rytioja et al., 2014).

The *L. crinitus* U9-1 strain also showed significant cellulolytic activity (Table 1); however, it was less efficient (than *L. edodes* U8-1 and *S. commune* U6-7) in degrading carboxymethyl cellulose. Atri and Sharma (2012) reported that all types of *Lentinus* spp cultured in media containing carboxymethyl cellulose presented cellulolytic activity. However, no other studies have attempted to analyze the cellulase production capacity of *L. crinitus* so far.

Several of the cellulase-codifying genes detected in the strains analyzed in our study (Table 2), such as *cel*7A and *cel*6B, have already been reported in other basidiomycetes such as *Phanerochaete chrysosporium* (Muñoz et al., 2001) and *Cropinopsis cinerea* (Liu et al., 2009), respectively. Similarly, Ishihara et al. (2005) noted that the genes *cel*3A and *egl* (endoglucanase gene) identified (in their study) in *Polyporus arcularius* and *L. edodes* U8-1, respectively had been previously identified in other species of basidiomycetes such as *Ustilago maydis* (Zheng and Ding, 2013).

In this study, only the gene sequence of *cel*6B of *L. edodes* U8-1 was analyzed; additionally, its characteristic cellulase protein domains, such as the glyco_hydro_6 domain (Table 4), which was representative of the cellulase-expressing functionality of this gene, were identified. This gene was previously isolated from *L. edodes* by Taipakova et al. (2011), and the sequence of amino acids codified by the gene was highly homologous to the protein sequences of the glycosyl hydrolase family (No. 6). Lee et al. (2001) reported that the gene *cel*6B from *L. edodes* contains 444 amino acids and has a typical catalytic domain that can be classified as glycosyl hydrolase family 6 member (*cbh*II-1). Cellulolytic enzymes were classified into at least 120 protein families, and they constitute one of the largest groups in the modern structural classification of glycosyl hydrolases. Bhat and Bhat (1997), when analyzing cellulase catalytic domains, identified a considerable variation among cellulases and, by analyzing the hydrophobic groups of catalytic domains, classified them into structurally related families, where in the members of the same family presented the same protein configuration and stereo selectivity, and shared the same hydrolytic mechanism.

Family 6 of glycosyl hydrolases is mainly composed of proteins produced by cellulolytic bacteria and fungi (Liu et al., 2009). This family is composed of endoglucanases and cellobiohydrolases, and its members can vary according to their activity profiles. Endoglucanases and cellobiohydrolases present a distorted β/α-barrel; however, cellobiohydrolases contain an

active site enclosed by two loops that form a tunnel whereas endoglucanases have a single loop with their active site being located within a gap (Liu et al., 2009).

The results of this study contribute to the existing knowledge on cellulases in basidiomycetes, and serve as a basis for future studies on the expression of these genes, and characterization of the catalytic activity of these enzymes. This allows for better utilization of these fungi in degrading vegetal fibers from agro-industrial residues and in other biotechnological applications.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by a grant provided by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Universidade Paranaense, and the Postgraduate Program of Biotechnology Applied to Agriculture.

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