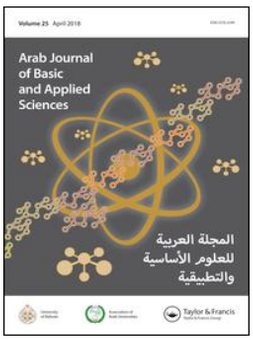

Arab Journal of Basic and Applied Sciences

ISSN: (Print) 2576-5299 (Online) Journal homepage: https://www.tandfonline.com/loi/tabs20

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To cite this article: Romina O. Coniglio, María I. Fonseca, Gabriela V. Díaz, Ornella Ontañon, Silvina Ghio, Eleonora Campos & Pedro D. Zapata (2019) Optimization of cellobiohydrolase production and secretome analysis of *Trametes villosa* LBM 033 suitable for lignocellulosic bioconversion, Arab Journal of Basic and Applied Sciences, 26:1, 182-192, DOI: 10.1080/25765299.2019.1598107

To link to this article: https://doi.org/10.1080/25765299.2019.1598107

Published online: 06 Apr 2019.

ARAB JOURNAL OF BASIC AND APPLIED SCIENCES
2019, VOL. 26, NO. 1, 182–192
https://doi.org/10.1080/25765299.2019.1598107

University of Bahrain
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ORIGINAL ARTICLE

Optimization of cellobiohydrolase production and secretome analysis of *Trametes villosa* LBM 033 suitable for lignocellulosic bioconversion

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ABSTRACT

The production of bioethanol from lignocellulosic biomass comprises the enzymatic hydrolysis of lignocellulosic structures by three major cellulases. Among them, cellobiohydrolases are considered to be key enzymes playing a significant role on cellulose degradation. The ability to produce lignocellulolytic enzymes by fungi such as *Trametes villosa* makes them appropriate degraders for large-scale applications. In this context, the aim of this study was to obtain and characterize a cellobiohydrolase-enriched extracellular extract of *T. villosa* LBM 033 (Misiones, Argentina), which is suitable for the enzymatic hydrolysis of lignocellulosic residues. The effect of carbon and nitrogen sources on cellobiohydrolase activity was evaluated using experimental designs and a culture medium was optimized to obtain a cellobiohydrolase-enriched extract suitable for the hydrolysis of lignocellulosic biomass. Moreover, by secretome analysis, nine enzymes involved in lignocellulosic biomass degradation were identified under the optimized conditions; among them is a cellobiohydrolase II from the glycosil-hydrolase 6 family.

ARTICLE HISTORY
Received 16 October 2018
Revised 8 March 2019
Accepted 15 March 2019

KEYWORDS
Cellobiohydrolases; central composite design; secretome; optimized culture medium; *Trametes villosa*

1. Introduction

Lignocellulose is the major component of biomass and the most abundant renewable organic resource on Earth. It consists of cellulose, hemicelluloses and lignin. Large amounts of lignocellulosic waste are generated through forestry and agricultural industries, and is widely recognized as an excellent low-cost and abundantly available feedstock for bioethanol production (Kumar, Gautam, & Dutt, 2016). Production of bioethanol from lignocellulosic biomass comprises hydrolysis to fermentable sugars, followed by fermentation and finally distillation. The hydrolysis process separates long chains of carbohydrate from cellulose with the addition of a water molecule which is a crucial stage, determining overall process efficiency. Hydrolysis is usually catalysed by enzymes or acids. Enzymatic hydrolysis is environment-friendly and, unlike acid hydrolysis, enzymes work in a mild environment, hence reducing equipment maintenance (Aditiya, Mahlia, Chong, Nur, & Sebayang, 2016). Therefore, the enzymatic hydrolysis of plant carbohydrates emerges as the most prominent technology for converting biomass into bioethanol (Van Dyk & Pletschke, 2012).

Enzymatic hydrolysis of cellulose is based on three major cellulolytic activities acting synergically: endo-1,4-beta-D-glucanase (EG, E.C.3.2.1.4), cellobiohydrolase (CBH, E.C.3.2.1.176 and E.C.3.2.1.91) and β-glucosidase (BGL, E.C.3.2.1.21) (Ben Hmad & Gargouri, 2017). In fungi, CBHI and CBHII belong to the glycosyl-hydrolase (GH) families 7 and 6, respectively, and are thought to be key enzymes for cellulose degradation to cellobiose (Takahashi et al., 2010).

Lignocellulolytic microorganisms, especially fungi, have attracted a great deal of interest as biomass degraders for large-scale applications due to their ability to produce large amounts of extracellular lignocellulolytic enzymes (Wang, Li, Fang, Wang, & Qu, 2012). In this sense, polypores (Polyporaceae, Agaricomycetes) such as *Trametes villosa* are in charge of wood rot in 96% of cases (Martínez-Anaya, Balcázar-López, Dantán-González, & Folch-Mallol, 2008).

Many successful attempts have been made to improve fungal lignocellulolytic activity. Nevertheless, the high cost of cellulase enzymes, more than 20% of second-generation ethanol production, still remains a challenge (Dashtban, Schraft, & Qin, 2009).

In general, in order to improve this technology and reduce the production costs, the efficiency

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Table 1. Independent variables and their levels for the screening experimental design.

Independent variables		Levels +1	Levels −1
Carbon sources	Crystalline cellulose CMC[a] Glucose Cassava bagasse Pine sawdust Sugarcane bagasse st	10 g l^{-1}	0 g l^{-1}
Nitrogen sources	Ammonium sulphate Peptone Yeast extract	1 g l^{-1}	0 g l^{-1}
Surfactant	Tween 80	1 ml l^{-1}	0 ml l^{-1}

[a]Carboximethyl cellulose.

Table 2. Experimental codes, ranges and levels of independent variables for the CCD.

Independent variables	Levels −1.35	−1	0	+1.35	+1
Crystalline cellulose (x_1)	8.23 g l^{-1}	10 g l^{-1}	15 g l^{-1}	20 g l^{-1}	21.76 g l^{-1}
Ammonium sulphate (x_2)	1.647 g l^{-1}	2 g l^{-1}	3 g l^{-1}	4 g l^{-1}	4.353 g l^{-1}
Peptone (x_3)					

optimization of cellulase production and the identification and characterization of new enzymes such as CBHs have become a priority in bioethanol technology research.

The aim of this study was to obtain and characterize a CBH-enriched extracellular extract of *T. villosa* LBM 033, a native isolate from Misiones (Argentina), suitable for the enzymatic hydrolysis of lignocellulosic residues.

2. Materials and methods

2.1. Fungi material and cultivation

The local strain *T. villosa* LBM 033 was used. This strain was deposited in the collection of the Molecular Biotechnology Laboratory, Misiones Biotechnology Institute "María Ebe Reca", National University of Misiones. The strain was maintained in MEA medium (12.7 g l^{-1} malt extract, 15 g l^{-1} agar) at 28 °C under static conditions until its mycelial development and conserved at 4 °C. *T. villosa* LBM 033 was cultivated in 100-ml Erlenmeyer flasks containing 20 ml of Czapek medium (2 g l^{-1} $NaNO_3$, 1 g l^{-1} KH_2PO_4, 0.5 g l^{-1} KCl, 0.5 g l^{-1} $MgSO_4{\cdot}7H_2O$, 0.01 g l^{-1} $FeSO_4{\cdot}7H_2O$) supplemented with concentrated nutrients according to the factorial (Table 1) and central composite design (CCD) (Table 2). The pH was adjusted to 4.8 with 100% glacial acetic acid. After inoculation with 5 mm agar plugs covered with young mycelium, the Erlenmeyer flasks were incubated at 28 ± 2 °C under mixing at 100 rpm for 12 days. After this period, the culture broths were centrifuged at 10,000 *g* for 10 min at 4 °C to obtain cell-free supernatants.

2.2. CBH activity

CBH activity of the cell-free supernatants was assayed following a modified protocol described by Wu, Zhao, and Gao (2006). The reaction mixtures containing 200 µl of cell-free supernatants and 800 µl of 500 µM ᵖ-nitrophenyl-β-D-cellobioside (ᵖNPC; Sigma, MO, USA) in 0.05 M sodium acetate buffer (pH 4.8) were incubated for 30 min at 50 °C. The amount of ᵖ-nitrophenol released was measured at 410 nm after adding 10% Na_2CO_3 to the reaction mixtures. One unit (U) of ᵖNPC-hydrolysing activity was defined as the amount of enzyme necessary to release 1 µmol of ᵖ-nitrophenol per minute of reaction.

2.3. Screening of factors affecting CBH activity

To screen the key ingredients significantly affecting CBH activity of *T. villosa* LBM 033, a 1/32 fractional factorial design was employed using the software *Statgraphics Centurion XVI.I*.

A total of 10 factors including different carbon sources, nitrogen sources and Tween 80 were evaluated at two levels (−1 for low level and +1 for high level) in 66 experimental runs in addition to two runs at their centre points (Table 1).

The effect of each nutritional component on CBH activity was calculated according to the following equation:

$$E_i = \sum R_{i+} - \sum R_{i-}/N \quad (1)$$

where E_i is the effect of the nutritional component i under study; R_{i+} and R_{i-} are the responses of the dependent variable (CBH activity) of the experimental runs, with the nutritional components at the upper and lower levels, respectively; and N is the total number of experimental runs.

2.4. Central composite design

To see the effect of crystalline cellulose, peptone and ammonium sulphate on CBH activity, an orthogonal CCD at five levels (−1.35, −1, 0, +1, +1.35) was employed using the statistical software *Statgraphics Centurion XVI.I* (Table 2).

A CDD with three replicates at the centre point resulting in a total of 17 experiments covering the entire range of variable combinations was used to optimize the selected key variables for CBH activity. The behaviour of the system was explained by the following equation:

$$y = a_0 + \sum a_i x_i + \sum a_{ii} x^2{}_i + \sum a_{ij} x_i x_j \quad (2)$$

where y represents response variable (CBH activity); a_0 is the interception coefficient; a_i is the coefficient of the linear effect; a_{ii} is the coefficient of quadratic effect; and a_{ij} is the coefficient of interaction effect.

Table 3. Factorial design matrix for 10 variables and CBH activity (U l^{-1}) for 68 experimental runs.

Run N°	Cellulose	Glucose	CMC[a]	Pine sawdust	Sugarcane bagasse	Cassava bagasse	Ammonium sulphate	Peptone	Yeast extract	Tween 80	**CBH activity (U l^{-1})**
1	−1	−1	−1	−1	−1	1	1	1	1	1	5.44
2	1	−1	−1	−1	−1	−1	−1	−1	−1	1	0.6
3	−1	1	−1	−1	−1	−1	−1	−1	1	−1	3.92
4	1	1	−1	−1	−1	1	1	1	−1	−1	0.12
5	−1	−1	1	−1	−1	−1	−1	1	−1	−1	13.74
6	1	−1	1	−1	−1	1	1	−1	1	−1	9.49
7	−1	1	1	−1	−1	1	1	−1	−1	1	2.12
8	1	1	1	−1	−1	−1	−1	1	1	1	57.16
9	−1	−1	−1	1	−1	−1	1	−1	−1	−1	0.34
10	1	−1	−1	1	−1	1	−1	1	1	−1	11.38
11	−1	1	−1	1	−1	1	−1	1	−1	1	4.1
12	1	1	−1	1	−1	−1	1	−1	1	1	0.16
13	−1	−1	1	1	−1	1	−1	−1	1	1	16.07
14	1	−1	1	1	−1	−1	1	1	−1	1	66.55
15	−1	1	1	1	−1	−1	1	1	1	−1	35.16
16	1	1	1	1	−1	1	−1	−1	−1	−1	0.03
17	−1	−1	−1	−1	1	1	−1	−1	−1	−1	0.41
18	1	−1	−1	−1	1	−1	1	1	1	−1	69.36
19	−1	1	−1	−1	1	−1	1	1	−1	1	34.07
20	1	1	−1	−1	1	1	−1	−1	1	1	2.38
21	−1	−1	1	−1	1	−1	1	−1	1	1	19.43
22	1	−1	1	−1	1	1	−1	1	−1	1	2.11
23	−1	1	1	−1	1	1	−1	1	1	−1	3.43
24	1	1	1	−1	1	−1	1	−1	−1	−1	8.9
25	−1	−1	−1	1	1	−1	−1	1	1	1	7.27
26	1	−1	−1	1	1	1	1	−1	−1	1	0.18
.28	1	1	−1	1	1	−1	−1	1	−1	−1	23.8
29	−1	−1	1	1	1	1	1	1	−1	−1	15.57
30	1	−1	1	1	1	−1	−1	−1	1	−1	31.96
31	−1	1	1	1	1	−1	−1	−1	−1	1	0.3
32	1	1	1	1	1	1	1	1	1	1	34.17
33	0	0	0	0	0	0	0	0	0	0	19.34
34	0	0	0	0	0	0	0	0	0	0	15.52
35	−1	−1	−1	−1	−1	1	1	1	1	1	37.49
36	1	−1	−1	−1	−1	−1	−1	−1	−1	1	0.26
37	−1	1	−1	−1	−1	−1	−1	−1	1	−1	0.19
38	1	1	−1	−1	−1	1	1	1	−1	−1	0.11
39	−1	−1	1	−1	−1	−1	−1	1	−1	−1	6.46
40	1	−1	1	−1	−1	1	1	−1	1	−1	85.42
41	−1	1	1	−1	−1	1	1	−1	−1	1	1.92
42	1	1	1	−1	−1	−1	−1	1	1	1	11.36
43	−1	−1	−1	1	−1	−1	1	−1	−1	−1	2.3
44	1	−1	−1	1	−1	1	−1	1	1	−1	19.41
45	−1	1	−1	1	−1	1	−1	1	−1	1	2.15
46	1	1	−1	1	−1	−1	1	−1	1	1	0.11
47	−1	−1	1	1	−1	1	−1	−1	1	1	11.55
48	1	−1	1	1	−1	−1	1	1	−1	1	46.63
49	−1	1	1	1	−1	−1	1	1	1	−1	9.32
50	1	1	1	1	−1	1	−1	−1	−1	−1	0.01
51	−1	−1	−1	−1	1	1	−1	−1	−1	−1	0.14
52	1	−1	−1	−1	1	−1	1	1	1	−1	81.7
53	−1	1	−1	−1	1	−1	1	1	−1	1	33.06
54	1	1	−1	−1	1	1	−1	−1	1	1	3.15
55	−1	−1	1	−1	1	−1	1	−1	1	1	19.43
56	1	−1	1	−1	1	1	−1	1	−1	1	24.43
57	−1	1	1	−1	1	1	−1	1	1	−1	3.58
58	1	1	1	−1	1	−1	1	−1	−1	−1	9.69
59	−1	−1	−1	1	1	−1	−1	1	1	1	8.67
60	1	−1	−1	1	1	1	1	−1	−1	1	0.19
61	−1	1	−1	1	1	1	1	−1	1	−1	1.05
62	1	1	−1	1	1	−1	−1	1	−1	−1	48.74
63	−1	−1	1	1	1	1	1	1	−1	−1	46.39
64	1	−1	1	1	1	−1	−1	−1	1	−1	36.45
65	−1	1	1	1	1	−1	−1	−1	−1	1	04.2
66	1	1	1	1	1	1	1	1	1	1	61.2
67	0	0	0	0	0	0	0	0	0	0	51.81
68	0	0	0	0	0	0	0	0	0	0	42.16

[a]Carboximethyl cellulose.

x_i and x_j denote the coded levels of variables X_i and X_j in the experiments.

The variable X_i was coded as x_i according to the following transformation equation:

$$x_i = (X_i - X_0)/\Delta X_i \quad (3)$$

where x_i is the dimensionless value of an independent variable; X_i is the real value of an independent variable; X_0 is the value of X_i in the central point; and ΔX_i represents the step of change of the variable.

Table 4. Analysis of variance (ANOVA) for CBH activity from screening experimental design. MS, mean sum of squares; SS, sum of squares; DF, degrees of freedom. *P* values less than 0.05 (> 95% confidence level) indicate that the model terms were statistically significant.

Source	SS	DF	MS	*F* value	*P* value
Ammonium sulphate (x_2)	2,421.26	1	2,421.26	7.05	0.0102
Crystalline cellulose (x_1)	2,370.59	1	2,370.59	6.90	0.0110
Peptone (x_3)	4,612.96	1	4,612.96	13.42	0.0005
Lack of fit	2,532.52	5	506.503	1.47	0.2121
Pure error	20,276.0	59	343.661		
Total	32,213.3	67			

2.5. Validation of the statistical model

To validate the results from the CCD six tests were carried out with the predicted conditions by the model. The supernatant obtained was used to determine the CBH activity, evaluate the effects of pH and temperature on CBH activity and identify the expressed proteins under these conditions.

2.6. Effects of pH and temperature on CBH activity

The effects of pH on CBH activity of the extract was studied at different pH values (3.0, 4.0, 4.8, 5.0, 6.0, 7.0, 8.0, 9.0 and 10) at 50 °C. The relative activity was expressed as 100% of maximum enzymatic activity. To determine enzyme stability as a function of pH, the supernatant was incubated at different pH values (3.0, 4.8 and 7.0) at 50 °C. Residual activity was expressed a percentage, taking the initial enzymatic activity as 100%. The buffer solutions used were: 0.05 M citrate buffer for pH 3.0; 0.05 M sodium acetate buffer for pH 4.0 and 4.8 and 5.0; 0.05 M sodium phosphate buffer for pH 6.0 and 7.0 and 8.0; and 0.05 M Tris–glycine buffer for pH 9.0 and 10.0.

To evaluate the effect of temperature on CBH activity, the reaction mixture was incubated at pH 4.8 at the temperatures of 4, 10, 20, 30, 40, 50, 55, 60, 65, 70 and 80 °C while keeping all other variables constant. The relative activity was expressed as 100% of maximum enzymatic activity.

To evaluate the thermal stability, the supernatant was incubated at a number of temperatures (30, 50, 60 and 70 °C). At selected time intervals, the supernatant was removed and CBH activity was measured. Residual activity was expressed as a percentage, taking the initial enzymatic activity as 100%.

Fisher's minimum significant difference procedure (LSD) was used to discriminate between the means using *Statgraphics Centurion XVI.I* and the plots were graphed with *GraphPad Prism 5.01*.

2.7. Identification of secreted proteins

T. villosa LBM 033 was grown under the CCD optimized conditions for 12 days. Forty-five millilitres of the broth culture were then centrifuged at 10,000 *g* for 20 min at 4 °C and filtered through a 0.2-μm polyether-sulfone filter unit (Thermo Scientific, USA).

Proteins were precipitated with 5 ml of 100% trichloroacetic acid (TCA) overnight at 4 °C, washed with 100% acetone four times and with 100% ethanol three times. Dry protein powder was dissolved in 500 μl of water (HPLC grade), reduced with 10 mM dithiothreitol for 45 min at 56 °C and alkylated with 20 mM iodacetamide for 40 min in darkness. A second protein precipitation step was performed using 100% TCA at −20 °C for 2 h and then centrifugation at 10,000 *g* for 10 min. The obtained pellet was washed with cold acetone three times, centrifuging between washes in order to remove the TCA. After acetone evaporation, the pellet was stored at −20 °C until analysis by nano LC MS/MS at the Proteomics Core Facility CEQUIBIEM (Faculty of Exact Sciences, University of Buenos Aires/CONICET).

Proteolysis was performed with trypsin in 50 mM ammonium bicarbonate at pH 8 overnight. The samples were lyophilized and resuspended with 10 μl of 0.1% formic acid. Proteolytically cleaved peptides were separated prior to mass spectrometric analyses by nano HPLC (EASY-nLC 1000, Thermo Scientific, USA) using a reverse-phase column (EASY-Spray Accucore, Thermo Scientific, USA) at 35 °C with a pre-column (Acclaim PepMap, Thermo Scientific, USA). A gradient with two solutions was used: solvent A was aqueous solution in 0.1% formic acid and solvent B was acetonitrile in 0.1% formic acid. Separated peptides were monitored using Q-Exactive Orbitrap mass spectrometer (Thermo Scientific, USA) equipped with a nano electrospray ion source. The applied electrospray voltage was 3.5 kV.

The identity of each peptide was inferred using Proteome Discoverer (Thermo Scientific v. 1.4). MS/MS-spectral data were searched against the database of the draft genomic sequence of *T. versicolor* (Floudas et al., 2012). Only proteins with ≥2 unique peptides were considered as reliably identified.

3. Results

3.1. Screening of significant medium components for CBH activity

Because CBH production was maximum on day 12 after incubation, CBH activity was determined on the cell-free supernatant of *T. villosa* LBM 033 at this time (data not shown). The data among the 68 experimental runs indicated that there was a wide variation of CBH activity from 0.01 to 85.42 U l^{-1} (Table 3). This variation reflected the significance of the evaluated factors on enzyme activity.

Furthermore, given that the *P* value is greater than 0.05 for the lack of fit in the ANOVA table, the

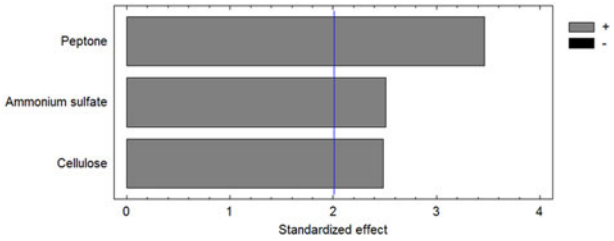

Figure 1. Pareto chart for CBH activity by *T. villosa* LBM 033. The length of the bars is proportional to the standardized effect of nutrients on CBH activity. Bars extending beyond the vertical line correspond to effects statistically significant at 95% confidence level.

model seems to be adequate for the data observed at the 95% confidence level (Table 4).

From all the screened factors, crystalline cellulose, peptone and ammonium sulphate had significant effect ($P \leq 0.05$) on CBH activity at the +1 level (Figure 1).

Excluding the variables which were non-significant, the model equation for CBH activity could be written as:

$$y(CBH\ activity) = 18.14 - 6.08\ {x_1}^2 - 6.15\ {x_2}^2 < 8.48\ {x_3}^2 \quad (4)$$

where y is the predicted response (CBH activity), and x_1, x_2 and x_3 are coded values for cellulose, ammonium sulphate and peptone, respectively.

Because these factors significantly affected CBH activity, crystalline cellulose, peptone and ammonium sulphate were chosen for the further optimization step.

3.2. CCD For optimization of CBH-enriched extract

From the screening experiment three nutritional factors (crystalline cellulose, peptone and ammonium sulphate) were found to play a significant role on CBH activity of *T. villosa* LBM 033 after 12 days of incubation. An orthogonal CCD 2^2 + star involving 17 runs was carried out in order to determine the optimal concentration for these nutritional compounds with the aim of obtaining the highest level of CBH activity (Table 5).

The quadratic expressions for both crystalline cellulose and nitrogen sources were significant ($P \leq 0.05$) on CBH activity, indicating that their optimal concentration values were around the central points. The R^2 value for the model, as the measure of the goodness of fit of the model, was 0.743, indicating that 74.3% of the total variability of CBH activity could be explained by the model (Table 6).

Response surface plots depicted the interaction between two variables for CBH activity by keeping the third one fixed at its middle concentration level. From these plots, the highest CBH activity was observed at the central points of the experimental design of all the factors (Figure 2).

The equation of the adjusted model representing the effect of each factor on enzyme activity after excluding the non-significant factors was given as the following:

$$y = 52,27 - 14,04\ {x_1}^2 - 19,01\ {x_2}^2 - 17,48\ {x_3}^2 \quad (5)$$

where y is the predicted response (CBH activity), and x_1, x_2 and x_3 are coded values for cellulose, ammonium sulphate and peptone, respectively.

On the other hand, the model predicted a maximum CBH activity of 52.2 U l^{-1} in 15 g l^{-1} of crystalline cellulose and 3 g l^{-1} of peptone and ammonium sulphate.

To validate the predicted model, six additional experimental runs were carried out with the optimized medium composition and the CBH activity of 49 ± 5 U l^{-1} was reached, showing agreement with the predicted value.

3.3. Effect of pH and temperature on CBH activity

The CBH activity in the optimized medium was evaluated at different pH values and temperatures.

The maximum CBH activity ($P < 0.05$) was found at pH 4.8, while at pH 4.0 and 5.0 the CBH activity remained between 80% and 90% (Figure 3a). Regarding the pH stability, enzymatic activity was stable above 50% until 72 h and then dropped dramatically after 96 h at pH 4.8 (Figure 3b) and about one-half of the initial CBH activity was retained after 6 h of incubation at pH 3.0 and 7.0 (Figure 3c).

The maximum enzymatic activity ($P < 0.05$) was found at 60 °C, with 58% of CBH activity at 50 °C (Figure 4 a). Regarding thermal stability, CBH activity was stable for 48 h above 50% at 30 °C and dropped below 50% after 1 h of incubation at 50 °C (Figure 4 b). The activity remained above 50% only for 1 min at 60 and 70 °C (Figure 4 c). It may be concluded from these observations that the extract should be used under moderate temperature conditions (below 50 °C) for the best CBH activity.

3.4. Characterization of secreted proteins under optimized conditions

The CBH-enriched extract of *T. villosa* LBM 033 was analysed by mass spectrometry. Because the *T. villosa* genome has not been sequenced to date, the *T. versicolor* genome was used as a reference for the prediction of secreted proteins by *T. villosa* LBM 033 under optimized conditions.

From the database search, 36 redundant peptides were identified, assigned to 17 proteins, where nine of them resulting in equal or more than two high

Table 5. Central composite design matrix and CBH activity (U l^{-1}).

Run N°	Ammonium sulphate (x_2)	Cellulose (x_1)	Peptone (x_3)	CBH activity (U l^{-1})
1	−1	−1	−1	0.28
2	−1	1	−1	4.88
3	1	−1	−1	10.77
4	1	1	−1	6.99
5	−1	−1	1	0.74
6	−1	1	1	16.01
7	1	−1	1	11.33
8	1	1	1	5.46
9	0	−1.35	0	24.52
10	0	1.35	0	5.36
11	−1.35	0	0	4.72
12	1.35	0	0	6.97
13	0	0	−1.35	16.49
14	0	0	1.35	0.8
15	0	0	0	71
16	0	0	0	62
17	0	0	0	51

Table 6. Analysis of variance (ANOVA) for CBH activity obtained from CCD. MS, mean sum of squares; SS, sum of squares; DF, degrees of freedom. *P* values less than 0.05 (>95% confidence level) indicate that the model terms were statistically significant.

Source	SS	DF	MS	*F* value	*P* value
Ammonium sulphate^2 $(x_2)^2$	2,423.39	1	2,423.39	15.77	0.0016
Crystalline cellulose^2 $(x_1)^2$	1,322.46	1	1,322.46	8.60	0.0116
Peptone^2 $(x_3)^2$	2,049.2	1	2,049.2	13.33	0.0029
Total error	1,998.02	13	153.694		
Total	7,793.03	16			

confidence peptides were considered as a primary condition to ensure the utmost confident identity (Table 7).

In general, proteins involved in lignocellulose biodegradation were detected in the *T. villosa* LBM 033 secretome. The main protein identified was a CBH of GH6, by matching two peptide sequences (VVAVIEPDSLANLVTNLNVQK and AASVANIPTFTWLDSVAK) belonging to CBHII of *T. versicolor* (Q9P8N1, Uniprot). This protein presents 436 amino acids, a pI of 5.83 and a molecular weight of 46.1 kDa. Its modular structure consists in a carbohydrate-binding module type 1 (CBM 1) from positions 8 to 34 and a glycol-hydro-6 catalytic domain from positions 96 to 402.

Furthermore, four peroxidases (manganese peroxidases, MnPs EC1.11.1.13, and lignin peroxidases, LiPs EC 1.11.1.14) and a cellobiose dehydrogenase (EC1.1.99.18) were found, which are involved in the decomposition of lignocellulosic components. Additionally, a putative chitinase (EC 3.2.1.14) belonging to the GH18 family, an extracellular metalloproteinase (EC 3.4.24) and a hypothetical protein were detected. In total, 66% of the secreted proteins identified by the *T. villosa* LBM 033 extract under the optimized culture conditions could be associated to lignocellulosic biomass degradation.

4. Discussion

CBHs are highly attractive enzymes for the deconstruction of recalcitrant crystalline cellulose, which

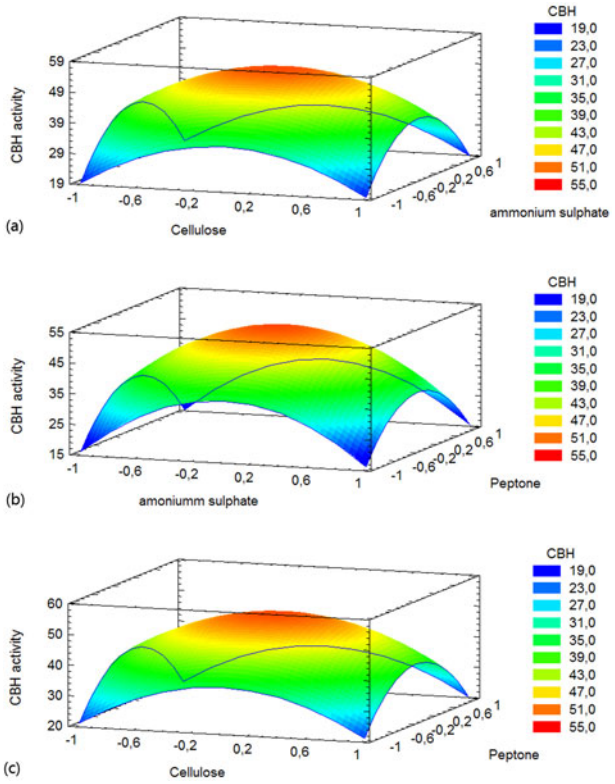

Figure 2. Three-dimensional response surface plots for CBH activity by *T. villosa* LBM 033. The plots show the interactive effects of medium components. (a) Cellulose and ammonium sulphate when peptone is fixed on its middle level, (b) ammonium sulphate and peptone when cellulose is fixed on its middle level and (c) cellulose and peptone when ammonium sulphate is fixed on its middle level. CBH activity is shown in U l^{-1}.

constitutes a challenge in biomass hydrolysis. In particular, fungal CBHs are of great interest because potent cellulolytic fungi are able to produce two different forms of CBH enzymes: CBHI and CBHII (Chukeatirote et al., 2012), which collectively represent the main components of the cellulase complex, as reported for *Trichoderma reesei* (Uusitalo, Nevalainen, Harkki, Knowles, & Penttilä, 1991) and *Volvariella volvácea* (Jia, Dyer, Buswell, & Peberdy, 1999).

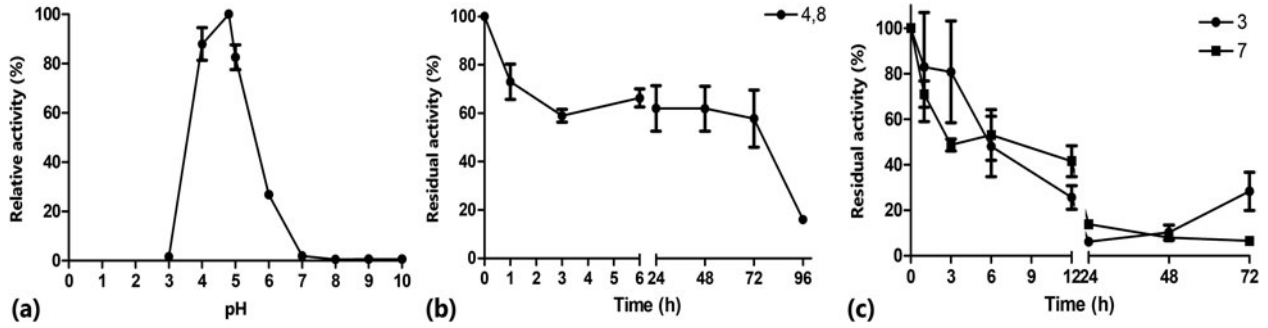

Figure 3. Effect of pH on CBH activity in CBH-enriched culture supernatant from *T. villosa* LBM 033. Influence of pH on CBH activity at 50 °C (a) and enzymatic stability at pH 4.8 (b), pH3.0 and pH 7.0 (c). The data are the mean ± standard deviation of duplicates.

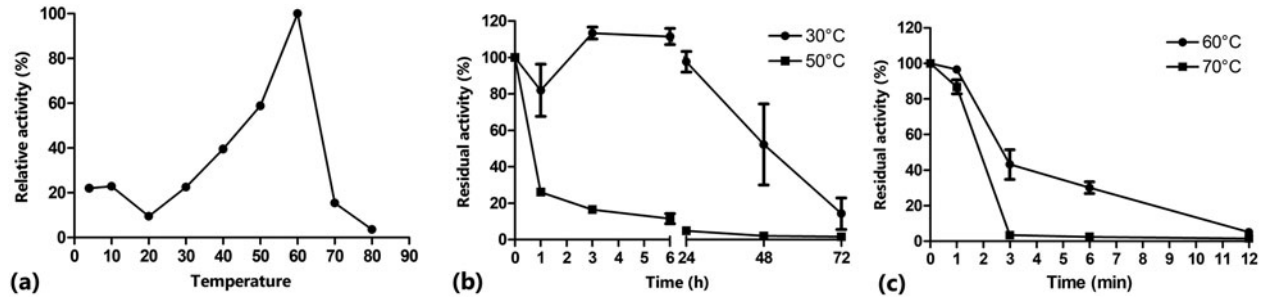

Figure 4. Effect of temperature on CBH activity in CBH-enriched culture supernatant from *T. villosa* LBM 033. Influence of CBH activity at different temperatures at pH 4.8 (a) and enzymatic stability at 30 °C and 50 °C (b), 60 °C and 70 °C (c). The data are the mean ± standard deviation of duplicates.

Table 7. Proteins identified in the CBH-enriched culture supernatant of *T. villosa* LBM 033 through mass spectrometry. MW, molecular weight; aas, amino acids.

Accession N°	Function description	Total peptides	Unique peptides	Peptides validated by MS/MS	MW (kDa)	aas	pI
Q9P8N1	Cellobiohydrolase GH6	2	2	VVAVIEPDSLANLVTNLNVQK AASVANIPTFTWLDSVAK	46.1	436	5.83
Q6B6M9	Peroxidase	4	3	LTFHDAIGISPAIAR TACEWQSFVNNQAK LNVFIGR LNVFIGRK	38.3	364	5.06
R7S9H1	Extracellular metalloproteinase	3	3	VNAFYIVNSIHDITYK MFLWDLTSPQR MFLWDLTSPQR DAWIQADANR	63.9	601	5.48
R7S863	Uncharacterized protein	3	3	KGDLATPLVLASGFDILLGR DVFVPPVLYPHAGTVWTK GDLATPLVLASGFDILLGR	14.4	135	6.54
Q99057	Peroxidase	4	4	DATRPAPDGLVPEPFDTLEDVFAR ITFHDAISFSPAMEAR LDFFLGR LQTDHLLAR	39.3	368	4.93
Q6B6N0	Peroxidase	4	3	KDATQPAPDLTVPEPFDDVSK DATQPAPDLTVPEPFDDVSK TACEWQSFVNNQAK LDVFLGR	38.3	364	4.97
Q6KB26	Peroxidase	2	2	LVPLPSDSVTDILAR LQSDFLIGR	38.1	364	4.53
R7S7Q1	Possible chitinase GH18	3	3	YDLDGLDFDWEYPNK FINEAGLR TVLNLVSK	45.1	436	4.16
O42729	Cellobiose dehydrogenase	3	3	DNVLVTQVIR ALVNPWLTNSVDK IGVDAALNAK	81.4	768	5.22

In submerged fermentation various factors such as pH, temperature, agitation, carbon and nitrogen sources significantly influence the fungi cellulolytic activities. Hence, the formulation of suitable fermentation strategies is a key factor determining the efficiency of the production of cellulases by a fungus (Sajith, Priji, Sreedevi, & Benjamin, 2016).

In order to obtain better productivity, the nutritional components of the culture media of *T. villosa* LBM 033 were evaluated using a factorial design to

screen variables which significantly affect CBH activity and then their concentrations were optimized by a CCD, keeping all physical conditions constant. In the screening experiment, crystalline cellulose, peptone and ammonium sulphate were selected and their concentrations for the optimized culture medium were identified as 15 g l^{-1} of crystalline cellulose and 3 g l^{-1} of nitrogen sources.

Many studies have been conducted to optimize EG, BGL and FPase activities; however, there have been very few studies focusing on CBH activity optimization. On this point, Hanif, Yasmeen, and Rajoka (2004) found that CBHs production by *Aspergillus niger* showed carbon sources variation, being repressed even at low glucose concentrations and stimulated by other carbohydrates. In the present work, for the first time, we study the effect of carbon and nitrogen sources mentioned in Table 1 on CBH of *T. villosa* LBM 033 activity in submerged medium through statistical experimental designs.

A large number of fungi are reported in literature, which produce cellulases with different substrates (Kuhad et al., 2016). In general, there is a consensus about the requirement of crystallinity and complexity of carbon sources such as crystalline cellulose and CMC to yield higher cellulase production by fungi (Hanif et al., 2004; Niranjane, Madhou, & Stevenson, 2007; Lee et al., 2011).

In this study, the maximal CBH activity of *T. villosa* LBM 033 was obtained with crystalline cellulose as a carbon source because CBHs preferentially attack the crystalline regions of cellulose. The production of cellulases is subject to transcriptional regulation by the available carbon source. Genes are repressed in the presence of glucose and highly induced when cellulose, its derivatives or certain oligosaccharides are present (Saloheimo, Aro, Ilmén, & Penttila, 2000). However, cellulose is insoluble and cannot enter the cell. The proposed mechanism for induction is that fungi produce basal levels of cellulases and their activity on cellulose produces a soluble inducer that can enter the cell and effect induction (Carle-Urioste et al., 1997).

Many studies agree that nitrogen sources improve CBH production (Lee et al., 2011; Mahmood et al., 2013). Hanif et al. (2004) found that ammonium sulphate was one of the best nitrogen sources for cellulase production. Moreover, peptone was also suggested as an important factor in optimized submerged media for cellulase production by several fungal species like *A. niger* (Acharya, Acharya, & Modi, 2008). In our study, we found that both peptone and ammonium sulphate as nitrogen sources also improve the CBH activity of *T. villosa* LBM 033. This response may be due to the fact that the amino acids are readily available sources to be used by fungi for growth and to produce extracellular enzymes such as cellulases.

In the present work, we optimized the culture conditions to obtain a CBH-enriched supernatant given that specific activities of CBHs are lower than other enzyme components which hydrolyse the crystalline cellulose (Ye, 2012). Unlike the ascomycetes CBHs, the CBHs from basidiomycetes have been poorly explored for cellulose saccharification. For instance, after cultivation of the white-rot fungus (*Pleurotus ostreatus*) and the brown-rot fungus (*Laetiporus sulphureus*) on sugar cane bagasse for 42 days and 2% CMC for 24 days, respectively, Valadares et al. (2016) found very low CBHs level for *L. sulphureus* (0.11 I U mg^{-1}) and no activity for *P. ostreatus*. Similarly, Kuuskeri et al. (2015) measured lignocellulose-converting enzyme activities of 49 *Phlebia* (Polyporales, Basidiomycota) isolates during 21 days of cultivation on semi-solid liquid medium with milled spruce as the carbon source. For these isolates, CBH activities were marginal and the highest value was observed for *P. centrifuga* on day 41 of incubation, reaching only 9.4 U l^{-1}. In this study, we obtained 49 ± 5 U l^{-1} on day 12 of incubation, a remarkable result, as CBH activity is usually low in macro fungi.As pH and temperature are the main factors involved in the production of bioethanol, stable EGs, CBHs and BGLs are sought for enzymatic biomass conversion (Yennamalli, Rader, Kenny, Wolt, & Sen, 2013). We analysed the effect of these parameters on CBH activity of supernatants obtained from *T. villosa* LBM 033 and our results were similar to those found in *Agaricus arvensis*, for which optimum CBH activity was found at 65 °C and a half-life of 65 min was determined at 50 °C (Lee et al., 2011). CBH activity is likely to decrease rapidly due to denaturation of the enzymatic structure at higher temperatures Mahmood et al., 2013). On the other hand, the enzymatic activity of *T. villosa* LBM 033 remained stable at least for 6 h at all assayed pH values; in agreement with both, TvCel7 (*T. versicolor*) (Lahjouji et al., 2007) and CBHI from *Thermoascus aurantiacus* (Hong, Tamaki, Yamamoto, & Kumagai, 2003) were seen to be stable over a wide range of pH.

To characterize the secreted proteins under the optimized culture conditions for CBH activity, the secretome of *T. villosa* LBM 033 was analysed by LS MS/MS, referring to the *T. versicolor* genome (Floudas et al., 2012). Other novel techniques on detecting biomolecules using nanomaterials were cited by Fan, Zhou, Qiu, and Zhang (2018), Luo, Fan, Zhou, Zhang, and Mei (2019) and Xue et al. (2019).

Seventeen proteins were determined in total and nine of them presented at least two unique peptides, thus guaranteeing their identity (Borin et al.,

2015). In this context, 66% of the identified proteins secreted by *T. villosa* LBM 033 in the optimized conditions corresponded to enzymes related to lignocellulose degradation.

Moreover, the characterization of the secretome of *T. villosa* LBM 033 allowed us to verify the presence of a GH6 CBH similar to other extracellular CBHs II which were characterized in fungi (Shin, Kim, Jeya, Lee, & Kim, 2010). The presence of this enzyme could be related to the regulatory mechanisms of the production of cellulases, which are finely controlled by activation and repression, hence cellulases are known as inducible enzymes. Only in the presence of the substrate these enzymes are induced and repressed when easily utilizable sugars are available (Behera, Sethi, Mishra, Dutta, & Thatoi, 2017). Evidence of inactivation of adsorbed CBH I was presented in recent studies using atomic force microscopy by Igarashi et al (2009; 2011]. They observed that some CBHI were inactivated on a substrate surface, thus explaining the absence of CBH I in the secretome of *T. villosa* LBM 033.

Furthermore, four peroxidases (MnPs and LiPs) and one cellobiose dehydrogenase were identified in the extracellular extract. The production of peroxidases seems to be a common feature of white rot fungi (Alfaro, Oguiza, Ramírez, & Pisabarro, 2014) and are limited to certain Agaricomycetes orders (Agaricales, Corticiales, Polyporales, Hymenochaetales). It is well known that laccase and peroxidase gene regulation is related to a hierarchy of environmental signals. Interestingly, the production of ligninolytic enzymes by the white-rot fungi depends on both carbon and nitrogen sources and their concentrations (Janusz, Kucharzyk, Pawlik, Staszczak, & Paszczynski, 2013).

5. Conclusion

We are focused on finding new native fungi to evaluate their capacity to produce enzymes for biotechnological applications, taking advantage of the outstanding biodiversity of our region. New enzymes are the foundation of tailor-made enzymatic complexes, an assembly designed to increase bioprocess efficiency. In this work we obtained a cellobiohydrolase-enriched cell-free extract of *Trametes villosa* LBM 033 (Misiones, Argentina) and the importance of this work lies in the potential of enzyme supernatants to be used in the enzymatic hydrolysis of lignocellulosic residues.

The CBH extracellular activity of *T. villosa* LBM 033 varies with the carbon and nitrogen sources present in the culture medium. In the present work, we optimized culture conditions to obtain a CBH-enriched extract suitable for the hydrolysis of lignocellulosic biomass in terms of which 66% of the secreted proteins identified by *T. villosa* LBM 033 corresponding to enzymes related to lignocellulose degradation, including a CBH II from the GH6 family.

In order to obtain a combination of a higher diversity of lignocellulolytic enzymes, more complex substrates can be used for fungal growth. For instance, the formulation of a combination of hydrolytic and oxidative enzymes could give even more favourable results in biotechnological applications such as bioethanol production.

Acknowledgements

The authors declare no potential competing interests. This work was partially funded by Secretary of Science and Technology of the National University of Misiones. MIF, PDZ and EC are CONICET (Argentina) researcher career members, ROC and GVD have PhD fellowships and OO holds a post-doctoral fellowship from CONICET (Argentina), and SG holds a fellowship from INTA.

Disclosure statement

No potential conflict of interest was reported by the authors.

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