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Solid-state production of cellulase by *Melanoporia* sp. CCT 7736: a new strain isolated from coconut shell (*Cocos nucifera* L.)

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Abstract

Background: The use of residues in industrial processing is expanding due to their low cost and abundant availability. Coconut shell is generated in large amounts in tropical areas due to the fruit processing and the coconut water consumption. In the present work, a new microbial strain was isolated from the coconut shell powder, molecularly identified as *Melanoporia* sp. CCT 7736 and applied for cellulase production in solid-state fermentation using the green coconut shell powder as substrate.

Results: The complete production process was optimized. Fermentation time was only 24 h, and the enzyme produced presented maximal activity at neutral pH (6.5) and 60 °C. The maximal enzyme activity after extraction optimization was 7.5 IU/gds (international units of enzyme activity per gram of dry solid). For the enzyme extraction, the rotation velocity, the extraction time, the temperature, and the solvent volume (buffer) were optimized using response surface methodology (RSM). The best results for the enzyme extraction were obtained at 250 rpm (orbital shaker) at 30 °C using 13.79 mL of a sodium acetate buffer (200 mM) at pH 6.5 after 10 min. Delignification pretreatment was not necessary since this fungus strain was able to degrade the lignin.

Conclusions: To the best of our knowledge, this work is the first report of cellulase production by *Melanoporia* sp. CCT 7736. Good results were obtained without the need for expensive pretreatment usually applied to lignocellulosic residues because the strain was isolated from coconut shell powder, and it is well adapted to this kind of substrate. The enzyme presented maximum activity at neutral pH instead of acidic pH as reported for the majority of industrial cellulases. The use of lignified coconut shell and the optimal pH at neutral values are the main advantages of the enzyme produced by *Melanoporia* sp. CCT 7736. In addition, the enzyme showed good stability during storage even at the crude broth and without any cryoprotection.

Keywords: Coconut green shell powder, Cellulase, *Melanoporia* sp. CCT 7736

Background

Large amounts of industrial residues are inadequately disposed in the environment generating pollution and other problems. Nowadays, the agricultural residues and their disposal are a concern due to the exponential increase in food industry. These residues are usually shells, seeds, peels, bagasse and other materials that might be used as raw material in industrial fermentations (Chandra et al. 2012). The annual coconut production is about 54 million tons, which results in approximately 19 million tons of fibers and shells (FAO 2012). The increase of agroindustrial residues stimulates the development of technological strategies to use these materials, changing their status from waste to raw material for use in production of valuable products (Foo and Hameed 2012).

Lignocellulose biomass, found in several agriculture residues, is the most abundant renewable resource in the word. Lately, this material has been considered for

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biotechnological applications. Cellulose is the most abundant polymer in nature and represents 50 % of the natural biomass (Wang et al. 2012). This biopolymer is composed of glucose units linked by β-(1-4) glucosidic bonds (Kinnarinen et al. 2012). Lignocellulose and lignin are usually found in cellulosic materials in different degrees depending on the biomass source (Wickramasinghe and Grzenia 2008). However, cellulosic materials are not directly fermentable, and a hydrolysis step is necessary to obtain simple sugars that can be metabolized by microorganisms (Singh et al. 2010).

Microbial cellulases that can be applied for lignocellulose saccharification have been extensively studied (Pavón-Orozco et al. 2012). However, the high hydrolysis costs are still a limiting factor for its industrial application (Cardona et al. 2010). Thus, the search for new enzymes might improve the productivity and reduce costs in making the lignocellulose hydrolysis on industrial scale (Cunha et al. 2012; Singhvi et al. 2011). As regards the search for new industrial enzymes, the use of new strains may be an alternative method to improve the hydrolysis process. The most studied microorganisms for cellulase enzyme production are fungi, especially from *Trichoderma* and *Aspergillus* genus.

The genus *Melanoporia* sp. CCT 7736 belongs to *Aphyllophorales* order, and it was first reported in 1907 by William Alphonso Murrill. This genus of fungi is reported as responsible for the brown rot in woods, and because of this property, it must be able to digest cellulose and hemicellulose materials, reducing the wood material to brownish cubic pieces (Kim et al. 2003; Ryvarden 1991; Samuelsson et al. 1994). The strain *Melanoporia castanea*, for example, was reported as lacase producer (Zhenguang and Minghao 2011). This enzyme is applied in several industrial processes including paper bleaching (Sigoillot et al. 2004). Also, the ability of *Melanoporia* genus to colonize *Quercus mongolica* and *Abies nephrolepis* trees has been reported by Zhou and Dai (Zhou and Dai 2012).

Despite the capacity presented by *Melanoporia* genus in lignocellulosic materials decomposition, the use of this strain for cellulase production was not reported, to the best of our knowledge. Thus, the objective of this study was to determine a protocol for the cellulase production in solid-state fermentation using a *Melanoporia* sp. CCT 7736 strain isolated from coconut shell waste.

Methods

Fungal strain isolation

Initially, three filamentous fungi were isolated from the coconut shell waste. Afterward, they were screened as cellulase producers. For this purpose, strains were cultivated on an orbital shaker in an Erlenmeyer's flasks containing 100 mL of the culture medium (potato dextrose broth), at 30 °C and 200 rpm of agitation. After 48 h, 1 mL of the culture was seeded in Petri dishes containing cellulose agar and incubated at 30 °C for 120 h. Cellulose agar was composed of KCl (0.5 g/L), $NaNO_3$ (3.0 g/L), $FeSO_4.7H_2O$ (1.0 g/L), K_2HPO_4 (1.0 g/L), agar (15.0 g/L), and (5.0 g/L). Fungi that showed halo-forming colonies were selected as cellulase producers, from which the best cellulase producer strain was chosen.

Molecular identification

The best cellulase producer strain was preliminarily identified by morphological analysis under light microscopy (40×). For molecular identification of the isolated strain, DNA extraction was done according to the methodology described by Raeder and Broda (Reader and Broda 1985). The ITS (internal transcribed spacers) regions were amplified by PCR (polymerase chain reaction) using as template the genomic DNA extracted from the sample. The PCR primers used were ITS-1 and ITS-4 (homologous to the ITS terminations). The amplified fragments were purified and sequenced using the automatic MegaBase 1000 equipment (GE Healthcare). The partial sequences obtained were grouped in a counting and compared to the ones found in GenBank and CBS. CLUSTAL X software was used to align the sequences (Thompson et al. 1994). Phylogenetic analysis was carried out using the software MEGA 4.0 (Tamura et al. 2007). The matrices distance were calculated using Kimura's model (Kimura 1980), and the phylogenetic tree was built using the Neighbor-Joining method (Saitou and Nei 1987). The bootstrap values were calculated from 1.000 resampling, using the software MEGA 4.0.

Coconut shell powder preparation

The coconut shell powder was obtained from dried shells according to the methodology described by Rodrigues et al. (2008). The powder was stored at room temperature (30 °C) in closed plastic bags (polyethylene) before use.

Coconut powder delignification

For the above purpose, 30 g of coconut shell powder was mixed with 100 mL of a 4 % (*w/v*) NaOH solution. The mixture was autoclaved at 121 °C for 30 min. After cooling to 30 °C, the coconut shell powder was washed with distilled water until the wash water presented pH 7.0. The coconut shell powder was then dried in an oven at 60 °C for 5 h (Pacheco et al. 2010) and applied as substrate for cellulase production by *Melanoporia* sp. CCT 7736.

Solid-state culture medium

Coconut shell powder and wheat bran were used as substrates. To determine the best proportion of coconut shell

powder and wheat bran, the amount of wheat bran was kept constant at 1.5 g, and the concentration of coconut shell powder was changed as presented in Table 1. After choosing the best substrate proportion (1.5 g of coconut shell powder and 1.5 of wheat bran), a saline solution containing 9 g/L of $(NH_4)_2SO_4$ and 1 g/L of KH_2PO_4 was used to humidify the solid mixture (3.0 g of total solids in 3.5 mL of saline solution).

Fermentation time

The culture medium's composition was set at 1.5 g of coconut shell powder, 1.5 of wheat bran, and 3.5 mL of saline solution (previous described). The pH was adjusted to 5.5 with acetic acid (0.1 N). Then, the medium was autoclaved at 121 °C for 5 min and cooled until 30 °C. Afterward, 1 mL of the inoculum containing 1×10^6 spores were added to the culture medium developed. The solid culture was incubated statically in Erlenmeyer's flasks in a B.O.D (Biochemistry Oxygen Demand) incubator at 30 °C during 120 h. Samples were taken every 24 h.

Enzyme activity assay

After the enzyme extraction, the solids and the microorganism were separated from the fermented extract by filtration. The substrates used for enzyme activity determination were: microcrystalline cellulose (Avicel®, Fluka), filter paper, xylanase, carboxymethyl cellulase (CMCase), and β-glucosidase (cellobiase).

The exoglucanase activity was determined using the crude enzyme extract (125 µl) mixed to the substrate (125 µl) composed of sodium acetate buffer (200 mM) containing 1 % (*w/v*) of Avicel solution. The reaction was interrupted by adding 250 µl of 3,5-dinitrosalicylic acid reagent (DNS).

The filter paper activity (FPase) was evaluated according to the Ghose (Ghose 1987). Five hundred microlitre of the sodium acetate buffer solution (200 mM and pH 6.5) was mixed with 250 µl of enzyme extract in a glass tube containing filter paper strips of Whatman No. 1 Size 1×6 cm. The mixture was incubated for 1 h at 60 °C. The reaction was stopped by the addition of DNS. The concentration of reducing sugar was measured according to the DNS method (Miller 1959).

The xylanase activity was determined according to Biely (Biely 1997). To 125 µL of the enzyme extract was mixed with 125 µL of the activity solution consisting of buffer (pH 6.5) containing 1 % (*w/v*) of xylan from birch wood (Fluka). The mixture was incubated for 1 h at 60 °C. The reaction was stopped by adding 250 µL of the DNS reagent.

For CMCase (endoglucanase) activity, 125 µL of enzyme extract and 125 µL of the activity solution consisting of buffer (pH 6.5) containing 1 % (*w/v*) of carboxymethylcellulose (Sigma) were mixed in a glass tube. The mixture was incubated for 1 h at 60 °C. The reaction was stopped by adding 250 µL of DNS reagent.

Cellobiase activity was determined mixing 125 µL of enzyme extract and 125 µL of the assay solution consisting of buffer (pH 6.5) containing 1 % (*w/v*) of cellobiose (Fluka). The mixture was incubated for 1 h at 60 °C. The reaction was stopped by adding 250 µL of DNS.

The results were expressed as international enzyme unit (IU) per gram of solid substrate (IU/gds). One IU is defined as the amount of enzyme that releases 1 µmol of reducing sugar per minute at the assay conditions (Binod et al. 2007).

Effect of temperature and pH on the cellulase activity

A central composite factorial design with three central points was built to evaluate the effect of the pH and the temperature on cellulase activity. The temperature was changed from 35.8 to 64.1 °C and extraction buffer pH from 4.3 to 5.7 (Table 2). Solid-state fermentation was carried out as previously described. Enzyme extraction is described further on. The experimental design was followed by a single factor optimization protocol where only the buffer pH was changed from 4.5 to 8.5 due to the positive effect of the pH on the enzyme effect.

Table 1 Solid-state culture medium for cellulase production by *Melanoporia* sp.

Coconut shell powder (g)	Wheat bran (g)	Saline solution (ml)	Enzyme activity (IU/gds)
0.5	1.5	2.32	1.80 ± 0.05
1.0	1.5	2.90	1.77 ± 0.04
1.5	1.5	3.48	2.46 ± 0.10
2.0	1.5	4.06	1.90 ± 0.08
2.5	1.5	4.64	1.85 ± 0.05
3.0	1.5	5.22	1.85 ± 0.03
0.0[a]	3.0	3.00	0.89 ± 0.04

[a] Wheat bran used as substrate for enzyme production

Table 2 Experimental planning (2^2) and results of enzyme activity as function of pH and temperature

Run	pH	Temperature (°C)	Enzyme activity (IU/gds)
1	4.5	40.0	0.11 ± 0.09
2	4.5	60.0	0.31 ± 0.10
3	5.5	40.0	0.55 ± 0.11
4	5.5	60.0	1.06 ± 0.01
5	4.3	50.0	nd
6	5.7	50.0	0.77 ± 0.07
7	5.0	35.8	nd
8	5.0	64.1	nd
9[a]	5.0	50.0	nd
10[a]	5.0	50.0	nd
11[a]	5.0	50.0	nd

[a] Central point

Nd non detected

Table 3 Experimental planning (2^2) and results of the enzyme activity as function of agitation and extraction time

Run	Agitation (rpm)	Time (min)	Enzyme activity (IU/gds)
1	50	10	2.09 ± 0.02
2	50	60	2.81 ± 0.06
3	200	10	3.05 ± 0.02
4	200	60	3.11 ± 0.01
5	50	35	2.51 ± 0.07
6	200	35	3.03 ± 0.09
7	125	10	2.43 ± 0.13
8	125	60	2.36 ± 0.29
9[a]	125	35	2.78 ± 0.15
10[a]	125	35	2.59 ± 0.06
11[a]	125	35	2.58 ± 0.01

[a] Central point

Optimization of the enzyme extraction from the fermented solid

A faced-center central composite design was carried out to optimize the enzyme extraction changing the rotation from 50 to 200 rpm and the extraction time from 10 to 60 min (Table 3). For enzyme extraction, 20 mL of the sodium acetate buffer was mixed with the solid medium (200 mM, pH 6.5). Afterward, the mixture was filtered through filter paper. The use of filter paper did not interfere with the enzyme activity. The enzyme activity was determined at the optimal pH and temperature previously determined (pH 6.5 and 60 °C). This first experimental design was followed by another one (central composite rotated design) where the buffer volume was changed from 13.79 to 56.21 mL, and the extraction time was changed from 5.86 to 34.14 min (Table 4). The extraction experiments were done at 25 °C.

Temperature effect on the enzyme stability during the storage

The crude extract was stored at −20 and 4 °C. The stored enzyme activity was performed every 30 days during 240 days (8 months). The results were analyzed regarding residual enzyme activity (%). The thermal stability was studied using the enzyme half-life time ($t_{1/2}$) calculated from Eqs. 1 and 2, which follows a zero-order kinetics:

$$Ar = Ar_0 - kt \quad (1)$$

Ar = relative activity (%)
Ar_0 = initial relative activity (%)
k = deactivation constant (months)
t = time (months)
where $t_{1/2}$ is

$$t_{1/2} = \frac{Ar_0}{2k} \quad (2)$$

Polyacrylamide gel electrophoresis (SDS-PAGE)

The protein profile of the crude enzyme extract produced by *Melanoporia* sp. CCT 7736, was submitted to electrophoresis using polyacrylamide gel (SDS-PAGE 12 %) at denatured and reducing condition at 150 V and 50 mA (Laemmli and Favre 1973). Samples collected at 0, 18, 24, and 72 h of fermentation were analyzed. The amount of sample loaded was 14 µl. Silver nitrate was used stain the protein bands. A Bench Mark™ Protein Ladder (Invitrogen, USA) was used as molecular mass marker.

Statistical analysis

The results were expressed as mean ± standard deviation. Experimental planning data were statistically evaluated by ANOVA analysis in Statistica 7.0 (Statsoft) software. When response surface methodology could not be applied, Tukey test was carried out for mean comparison at 5 % of significance.

Results and discussion

Fungal strain choice

The three strains isolated from the coconut shell waste were morphologically different. After 48 h of incubation in cellulose agar plates, where the only carbon source was cellulose, visible halo zones were observed in all isolates. This result indicated the ability of the isolates to hydrolyze the cellulose. Although the three isolated strains were able to degrade cellulose, one isolated showed better enzyme activity in submerse fermentation trials. This strain was selected and molecularly identified as *Melanoporia* sp. CCT 7736.

Table 4 Experimental planning (2^2) and results as function of buffer volume and agitation time

Run	Buffer volume (mL)	Time (min)	Enzyme activity (IU/gds)	Enzyme activity (IU/L)
1	13.79	20	7.50 ± 0.15	1630 ± 5
2	20	10	2.68 ± 0.08	1130 ± 3
3	20	30	4.21 ± 0.15	400 ± 5
4	35	5.86	1.20 ± 0.00	630 ± 2
5[a]	35	20	1.72 ± 0.02	110 ± 3
6[a]	35	20	1.95 ± 0.18	150 ± 6
7[a]	35	20	1.43 ± 0.01	120 ± 2
8	35	34.14	2.34 ± 0.10	200 ± 3
9	50	10	1.25 ± 0.07	80 ± 2
10	50	30	1.63 ± 0.10	100 ± 3
11	56.21	20	3.84 ± 0.00	200 ± 3

[a] Central point

Selection of the culture medium for cellulase production by *Melanoporia* sp. CCT 7736

According to the results presented in Table 1, cellulase production decreased with the increasing amount of coconut shell powder, and the maximum enzyme production by *Melanoporia* sp. CCT 7736 (2.46 ± 0.07 IU/gds) was obtained when 1.5 g of coconut shell powder and 1.5 g of wheat bran were used as a solid medium. When only wheat bran was used, the production was 0.89 IU/gds. When only coconut shell was used, agglomerates were formed inhibiting the microbial growth. The coconut shell powder is solid with small-sized particles (mean diameter < 1 mm) that tends to form agglomerates when humidified forming a paste. Thus, coconut shell powder was mixed with wheat bran to prevent agglomeration and make the medium suitable for solid-state fermentation. Wheat bran presents a better affinity to retain water compared to other materials. Its utilization in solid-state fermentation contributes to a better water retention and oxygen diffusion due to its large particle size. Wheat bran also increased the medium's porosity allowing a more homogenous fungi growth on the substrate surface. Wheat bran also was utilized by Dogaris et al. (2009) who evaluated the influence of wheat bran's addition in a medium made from sugarcane straw for cellulase production using *Neurospora crassa* DSM 1129. The best result was obtained with the combination of the two solid matrices. The use of wheat bran as a solid substrate for cellulase production was also reported elsewhere as a suitable substrate (Deswal et al. 2011; Krishna 2005). However, the coconut shell powder strongly increased the enzyme activity when used in a proper proportion. Thus, the culture medium selected for the further experiments was composed of 1.5 g of coconut shell powder, 1.5 g of wheat bran, and 3.48 mL of saline solution.

Delignified coconut shell powder as substrate

Many studies have been reported using delignified lignocellulosic material as a preferred substrate for cellulase production. Therefore, an assay in the optimum conditions previously determined was carried out using non-treated coconut shell powder and pretreated with NaOH (lignin removal). The results obtained showed that the lignin present in the coconut shell powder did not affect the cellulase production. Literature shows that the genus *Melanoporia* can decompose lignin (Zhou and Dai 2012). Thus, this strain is an interesting alternative to cellulase production, since the delignification is an expensive, laborious, and time-consuming step.

Fermentation time optimization

Figure 1 shows the enzyme activity during the fermentation for cellulase production. It was observed that after 24 h of fermentation the enzyme activity slightly decreased reaching 90 % of the initial activity at 96 h of fermentation. Afterward, a total activity loss was observed at 120 h of fermentation.

The results showed a peak at 24 h and a reduction in that enzyme activity after 24-h fermentation can be attributed to lack of nutrients necessary for the metabolism of *Melanoporia* sp. CCT 7736 or due to the accumulation of secondary metabolites. The activity loss can also be attributed to decreased enzyme interaction with the substrate (e.g., due to the inactivation of the enzyme adsorbed) (Ye et al. 2014).

Dogaris et al. (2009) studied the fermentation of *N. crassa* DSM 1129 for cellulase production in the solid state during 10 days. The best fermentation processing time was 48 h. In this case, only 24 h was sufficient for a good enzyme activity. Ang et al. (2013) using a more complex culture medium to cultivate *Aspergillus fumigatus,* reported that the best production of cellulases was

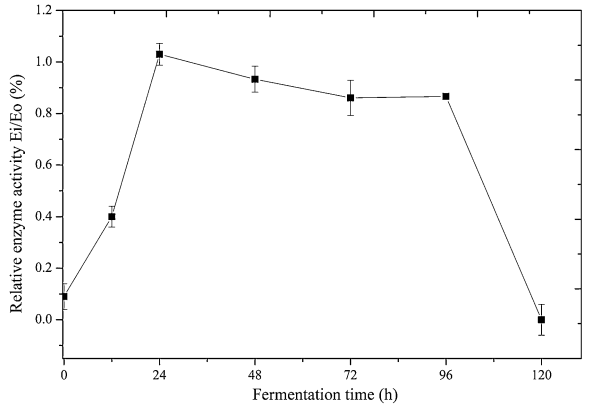

Fig. 1 Relative enzyme activity during the fermentation time in solid-state fermentation of *Melanoporia* sp. CCT 7736

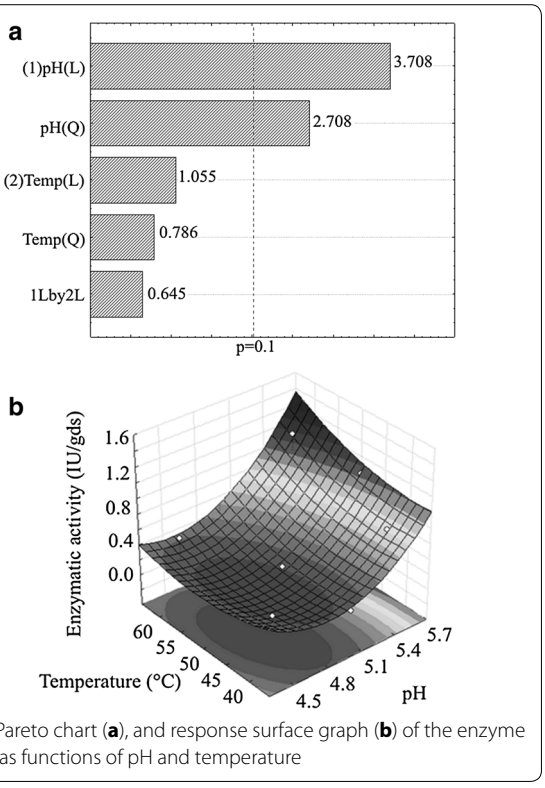

Fig. 2 Pareto chart (**a**), and response surface graph (**b**) of the enzyme activity as functions of pH and temperature

observed at 11 days of fermentation. In the present work, the best results were found at 24 h of fermentation, which is much shorter compared to other solid-state processing and, thus more interesting from the industrial point of view.

Effect of temperature and pH on the cellulase enzyme activity

The results obtained carrying out the experimental planning (Table 2) were fitted to the regression model presented in Eq. 3.

$$\text{Enzyme activity}\left(\text{IU/gds}\right) = 30.30 - 11.31\text{pH} + 1.11\text{pH}^2 - 0.14T + 0.0008T^2 + 0.02\text{pH} \times T \quad (3)$$

where pH = buffer pH, and T = temperature of the enzyme activity assay (°C).

The model was statistically validated by ANOVA analysis and F test at 90 % of confidence interval. The calculated F value was 5.0, which is greater than the listed one ($F_{5,5} = 3.45$). Thus, the model is statistically significant according to the F test, and the response surface methodology could be applied. Figure 2a shows the estimated effect of the independent variables (pH and temperature) on the enzyme activity. Only pH effect (linear and quadratic) was significant on enzyme activity. A maximal point could not be seen in the evaluated experimental domain. On the other hand, a minimum point was observed near to the central point with a tendency of activity increase with the pH increase. As pH produced a significant effect on enzyme activity, and no clear maximal point was observed in the response surface graph, a univariate essay was carried out at 60 °C by changing the pH from 4.5 to 8.5. According to the results showed in Fig. 3, lower amount of activity was found at higher and lower pH values, and the maximal enzyme activity was found at pH range from 6.0 to 6.5. This results contradicts the results published by Andrade et al. (2011) and Delabona et al. (2013), who reported optimum pH values at 3.0 for cellulases produced by *A. fumigatus* and *Trichoderma* sp. IS-05, respectively. Also, most of the commercial cellulases presents optimum activity in the acidic medium (pH < 5.0). A cellulolytic enzyme with optimum pH near to the neutral value, as obtained in the present work, is interesting for industrial application. A hydrolysis processing at high temperature carried out at neutral pH is less corrosive to the equipment (pipes and vessels) and generates a mild effluent with practically no need of pH correction before treatments.

Optimization of the enzyme extraction from the solid medium

The regression model obtained for the enzyme activity as a function of the rotation and extraction time according to the experimental planning (Table 3) is presented in Eq. 4.

$$\text{Enzyme activity}\left(\text{IU}/\text{gds}\right) = 2.0162 - 0.0042R + 0.00005R^2 + 0.0294t - 0.0002t^2 - 0.0001R \times t + 0 \quad (4)$$

where: t = extraction time (min) R = rotation (rpm).

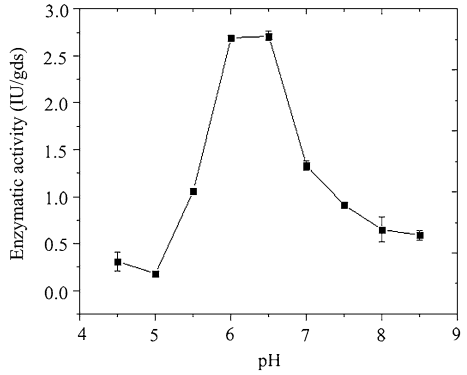

Fig. 3 Enzyme activity as the function of pH (60 °C)

The model was validated by ANOVA and F test at 95 % of confidence level. The calculated F value was 6.47, which is higher than the listed one ($F_{5,5} = 5.05$) at the given confidence interval. The regression coefficient was also satisfactory ($R^2 = 0.86$). Figure 4a shows the Pareto chart of the effects of the independent variables on evaluated the response. Only the linear effect of rotation was significant at the given confidence interval. According to the results, the increase in the rotation favored the enzyme exaction while extraction time had no influence. The time required for enzyme recovery depends on the enzyme solubility in the medium applied as extractor, which is influenced by rotation velocity (Gupta et al. 2008). According to the response surface graph presented in Fig. 4b, the rotation from 50 to 200 rpm resulted in an increase of the enzyme activity from 2.09–3.05 IU/gds at 10 min of extraction. To confirm the observed tendency, a univariate experiment was carried out fixing the extraction time at 10 min and increasing the rotation up to 250 rpm (range outside the experimental domain). The results obtained confirmed that when rotation increased, an increase on the enzyme activity was observed. According to the Tukey (95 % of confidence), the obtained values of enzyme activity at different rotations: 3.05 ± 0.01 IU/gds (200 rpm), 3.89 ± 0.08 IU/gds (225 rpm), and 5.05 ± 0.03 IU/gds (250 rpm) were statistically different. Thus, 250 rpm was chosen as the best value for the rotation system.

To evaluate the temperature effect on the enzyme extraction, another univariate experiment was carried out changing the extraction temperature from 10 to 30 °C. The results obtained for enzyme activity (4.09 ± 0.02 IU/gds at 10 °C, 4.27 ± 0.11 IU/gds at 20 °C and 4.56 ± 0.06 IU/gds at 30 °C), were not statistically different according to the Tukey test (95 % of confidence, $p < 0.05$). Thus, the temperature of 30 °C was chosen for the further assays, because this temperature is close to the room temperature.

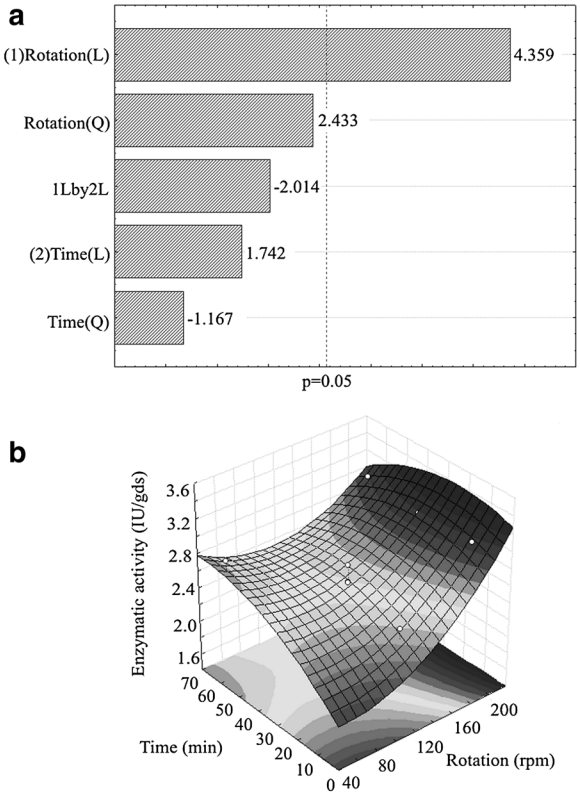

Fig. 4 Pareto chart (**a**), and response surface graph (**b**) of the enzyme activity as functions of rotation and extraction time

Gupta et al. (2008) reported that the solid-state fermentation processing for 15 min at 200 rpm and 35 °C resulted in the maximum extraction of endo-polygalacturonase. According to their results, the agitation improved the contact between the solid substrate and the solvent due to the breaking down of some solid agglomerates. Besides, agitation also improved the enzyme's liberation on the solution extraction.

Another important parameter in extraction processing is the concentration gradient because it affects the enzyme dissolution into the liquid medium. The concentration gradient is strongly affected by the solvent-to-solid ratio. Thus, to evaluate the effect of the amount of solvent added to the solid fermented substrate, another experimental process changing the solvent-to-solid ratio and the extraction time was carried out at 250 rpm and 30 °C. The regression model for the data obtained with this experimental design (Table 4) is presented in Eq. 5.

$$\text{Enzyme activity (IU/gds)} = 10.1177 + 0.2242t - 0.0028t^2 - 0.5577V + 0.0074V^2 - 0.0019t \times V + 0 \quad (5)$$

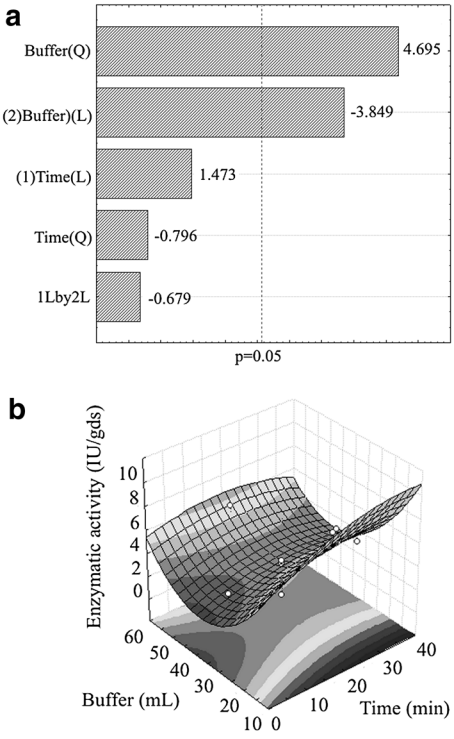

Fig. 5 Pareto chart (**a**), and response surface graph (**b**) of the enzyme activity as functions of buffer volume and time

where t = time (min); and V = buffer volume (mL).

The model was validated by ANOVA analysis and F test at 95 % of confidence interval. The model was statistically significant because the calculated F value (8.94) was higher than the listed one ($F_{5,5} = 5.05$) at the given confidence interval (95 %). The regression coefficient (0.89) was also satisfactory. Figure 5a shows the Pareto chart of the effect of the independent variables on the response. Figure 5b shows the results on the response surface graph. The extraction time was not significant, but the linear, and the quadratic effects of the buffer volume were significant on the enzyme activity. The figure shows that the extraction time was not significant on the enzyme recovery. The buffer volume that maximized the enzyme extraction from the solid medium was the lower applied volume (13.79 mL). Higher volumes resulted in lower enzyme activity due to the enzyme dilution.

Enzyme activity determination

After optimizing the enzyme extraction all enzyme activities comprising the cellulosic complex were determined. When xylan and CMC were used, the enzyme activities were 2.86 ± 0.18 and 2.80 ± 0.14 IU/gds, respectively, for each substrate. The filter paper activity was 3.05 ± 0.12 FPU/g. Cellobiase enzyme activity was not detected, and for avicel, the activity was 7.50 ± 0.15 IU/gds. In a study with *Aspergillus fumigatus* SK1, the cellulases production was found to be 3.36 FPU/g by solid-state fermentation. However, the enzyme production also depends on the chemical composition of the substrate, accessibility, and physiochemical association between its components (Gao et al. 2008).

Although *Melanoporia* presented the ability to decompose lignocellulosic substrates, there are no reports in the literature about the use of this strain in the cellulases production by fermentation processes. Within only 1 day (24 h), this strain showed the same cellulase production (3.05 ± 0.12 FPU/g) as that reported for *Aspergillus fumigatus* SK1 (3.36 FPU/g) after 11 days of fermentation.

Protein profile determination present in the crude enzyme (SDS-PAGE)

Figure 6 shows the electrophoretic profile of the crude enzyme produced by *Melanoporia* sp. CCT 7736. The results showed that the enzyme pool presents several bands with a molar mass ranging from 10 to 60 kDa. Bands corresponding to 24 h of fermentation were strongly stained, and bands corresponding to 0, 18, and 72 h showed lower intensity. These results suggest that the bands of 24 h correspond to a higher protein production, which is confirmed by the enzyme activity results previously reported in this study (Fig. 1).

Similarly, Dyka et al. (2009) analyzed the protein profile of a cellulolytic and hemicellulolytic system from *Bacillus licheniformis* with avicel and CMCase activities. They observed the presence of 14 bands ranging from 14 to 122 kDa. Annamalai et al. (2013) used lignocellulosic biomass as a substrate for *B. halodurans* CAS 1 cultivation for cellulase production. They estimated the molar mass of this enzyme to be 44 kDa. The author stated that this molar mass is much higher than that reported for cellulase from other *Bacillus* strains.

Cellulase stability during storage at low temperatures

The effect of storage temperature on the stability of the cellulases produced by *Melanoporia* sp. CCT 7736 is an important parameter for the commercial use of this enzyme. The inactivation profile and the enzyme half-life are shown in Fig. 7 and Table 5, respectively. The residual activity of the crude enzyme was 82 % at −20 °C and 71 % at 4 °C after 30 days. After 6 months at 4 °C, the enzyme lost almost all its activity, showing a sharp linear decrease with 2 % of residual activity. At −20 °C, the residual activity after 180 days was 44 % and after 8 months, 14 %. The enzyme half-life also was better at −20 °C temperature than at 4 °C. These results show a good stability of the enzyme produced by *Melanoporia* sp. CCT 7736, greater

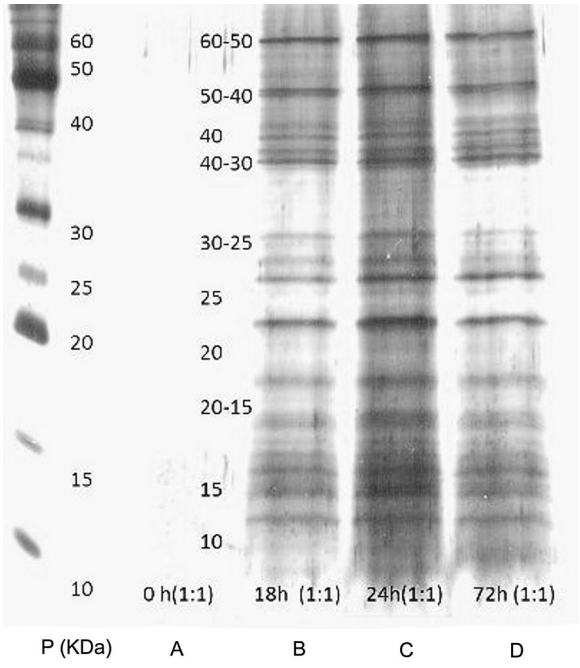

Fig. 6 SDS-PAGE of *Melanoporia* sp. CCT 7736 cellulolytic enzymes at different fermentation times. *P* is the molecular mass standard; *A*, *B*, *C*, and *D* are the fermentation samples at 0, 18, 24, and 72 h, respectively

than the values found in the literature, even when stored in the crude broth and without the presence of stabilizers. Stabilizers such as glycerol, for example, can increase the enzymatic stability (Singh et al. 1991; Kapoor and Kuhad 2008) and the enzyme half-life during storage. In another study, an endoglucanase from *T. viride* showed a

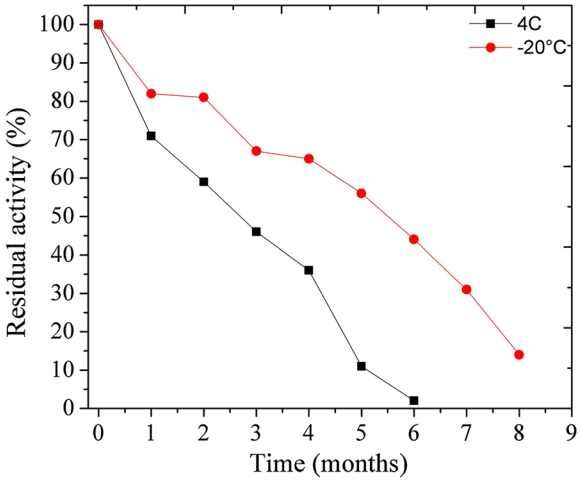

Fig. 7 Residual activity profiles during storage of the crude extracts of cellulase at 4 °C (*closed square*) and −20 °C (*closed circle*) for 8 months

Table 5 Half-life for the enzyme stored at 4 °C and −20 °C over time

Temperature (°C)	Half-life (months)	Zero-order kinetic constant (*k*)	Correlation coefficient (R^2)
4	3.12	0.16 ± 0.01	0.97
−20	5.55	0.09 ± 0.006	0.96

residual activity of 58 % after 30 days at 4 °C (Iqbal et al. 2011). These data show that the enzyme produced by *Melanoporia* sp. CCT 7736 exhibited superior stability compared to the results mentioned above.

According to Heller (Heller et al. 1997), freezing at −20 °C or −80 °C are the most common methods for enzyme cold storage. However, freezing may cause structural denaturation in enzyme solutions and loss of biological functions due to pH variations and "stress" (aggregation, unfolding, and dissociation) caused by the formation of ice crystals and freeze–thaw cycles, which reduce enzyme stability. However, for the enzyme produced by *Melanoporia* sp. CCT 7736, the freezing temperature of −20 °C was the most suitable. This stability is significant because this enzyme was able to remain stable even after 6 months of storage, while most of the enzymes present a stability for less than only 30 days.

The good stability presented by the cellulase produced by *Melanoporia* sp. CCT 7736 at freezing temperatures (−20 °C) is of interest. The enzyme can be stored crude without any prior pretreatment, which reduced its costs.

Conclusions

The new strain *Melanoporia* sp. CCT 7736 isolated from the green coconut shell powder was able to produce a good amount of cellulase at the optimized conditions (7.50 IU/gds) compared to the results published elsewhere for solid-state cellulase production. The short fermentation time (24 h) and pH (6.5) for the maximal enzyme activity is an additional advantage since the production processing is short, and the pH is adequate for the industrial purpose. No significant differences were found using the lignified and delignified substrates, which is another advantage of this fungi strain because delignification is a time-consuming and an expensive process. The molar mass characterization of the proteins with hydrolytic activity should be the object of further studies along with the protein sequence.

Authors' contributions

SLRO designed and performed the experiments along with the data analysis. SOS organized the manuscript draft and contributed to the experimental working. TCM designed and carried out the statistical optimization data for enzyme extraction. SR supervised the complete study along with experimentation, interpretation, and manuscript drafting. All the authors read and approved the final manuscript.

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SR is Professor of the Food Technology Department and of the Chemical Engineering Department at the Federal University of Ceará. SLRO conducted this study as a Ph.D. student in chemical engineering under SR's supervision. SOS both contributed as a post-doc within TCM working on cellulase applications.

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Competing interests
The authors declare that they have no competing interests.

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