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Saccharification of sugarcane bagasse using cellulases and oxidoreductases enzymes

Abstract

Lignin is the major polymer responsible for limiting the rates of lignocellulose degradation. Enzymatic hydrolysis of lignin (using oxidorreductases enzymes) open the way to action of cellulose and hemicellulose -degrading enzymes systems to obtain a fermentable hydrolysate rich in monomeric compounds from the carbohydrate polymers present in the biomass. *Trichoderma reesei* is an important fungus that is widely used as a source of cellulases for the hydrolysis of plant cell wall polysaccharides. In this study, was identified the filamentous fungi *Pestalotiopsis* sp BBF245, as a promising strain for production of oxidoreductases enzymes. Enzyme cocktails were composed, using cellulases from *T. reesei* and oxidoreductases enzymes from Pestalotiopsis sp BBF245 for hydrolysis of sugarcane bagasse (SCB) in natura. The amounts of reducing sugars released from the saccharification of bagasse was of 15,52 g/L after 24 hours of incubation. The results show that the combination of lignocellulose-degrading enzymes significantly increased the degradation of sugarcane bagasse and suggest that the use of enzyme cocktails oxidoreductases and cellulases may significantly improve the hydrolysis of biomass.

Keywords: *trichoderma*, pestalotiopsis, oxidoreductases, laccases, peroxidases, sugarcane bagasse

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Highlights

a. *Pestalotiopsis* sp BBF245 is a new enzymes source for biomass hydrolysis.

b. The interaction between cellullases enzymes from *Trichoderma reesei* and oxidorreductases enzymes from *Pestalotiopsis* sp BBF245 is being reportedThe combination of cellulases and oxidorreductases enzymes has positive effects and significantly increased the reducing sugar of SCB in 25%.

c. Potential biotechnological application of enzymes from Pestalotiopsis sp BBF245 was assessed.

Introduction

Application of enzymes to catalyze the degradation of biomass feedstocks is the most viable strategy to provide cost-efficient generation of fermentable monosaccharides. Globally, plants produce an estimated 200 billion tons of biomass per year in the form of sugars, polysaccharides, oils and other biopolymers, representing an unprecedented resource.[1,2] Vegetal biomass is considered a low-cost feedstock, available in massive quantities and can often be locally produced. The main chemical components of lignocellulosic biomass are cellulose (linear homogeneous structural polysaccharide composed of D-glucose units), hemicellulose (ramified heterogeneous structural polysaccharides composed of D-xylose, L-arabinose, D-mannose, D-galactose and D-glucose units), lignin (phenylpropanoid polymer composed of syringyl, guaiacyl and p-hydroxyphenyl units), pectin (ramified heterogeneous structural polysaccharides homogalacturonan, rhamnogalacturonan I, rhamnogalacturonan II, xylogalacturonan, apiogalacturonan mainly composed of D-galacturonic acid units), soluble sugars (D-glucose, D-fructose, sucrose and fructans), starch (linear or ramified homogeneous non-structural polysaccharide mainly composed of D-glucose), proteins and mineral compounds.[3,4]

The lignocellulose degradation is product of a concerted action of ligninases, cellulose and hemicellulose degrading enzymes systems and other accessories enzymes. Lignin is a phenolic polymer that constitutes a major barrier against cost-effective lignocellulosic biofuels by complexing with cellulose and preventing hydrolytic enzymes (cellulase, β-glucosidase) from accessing the sugar and by non-productively adsorbing such enzymes on the hydrophobic lignin surface.[5] Lignin can be degraded by means of extracellular enzymes as lignin peroxidases (EC 1.11.1.14), manganese peroxidases (EC 1.11.1.13), laccases (EC 1.10.3.2) and others, rendering the hemicellulose and cellulose more expose to enzyme attack.[6] Filamentous fungi are the preferred microorganisms on account of their production and secretion capacity of lignocellulosic enzymes for biomass degradation. However, the major bottleneck in the fungi selection for enzyme production is the need for a complex of enzymes, thus these fungi are able to secrete large or relatively low amounts of one or other enzyme and this ability can be influenced by different substrate composition and/or nutritional availability of carbon source.[7]

In Brazil, sugarcane is a prominent crop due to the success of sugar-based fuel ethanol, sugarcane harvest in 2017/2018 is expected to reach 647.6 million tons,[8] of which 161.9 million tons corresponding to bagasse, making it the most abundant agricultural residue produced in Brazil. The sugarcane cell wall shows an content of ~30% cellulose, ~50% hemicelluloses, and ~10% pectins.[9] Release of the sugars from the different plant polysaccharides requires a more complex set of carbohydrate-active enzymes. In addition to a number of cellulases, efficient enzymatic hydrolysis also needs synergistic activity of hemi cellulases (endoxylanases, β-xylosidase, α-arabinofuranosidase, and acetyl esterase), oxidorreductases (lignin peroxidases, manganese peroxidases, phenol oxidases, H_2O_2-producing enzymes, laccases, and β-etherases) and others auxiliary enzymes.

Optimization of enzyme mixtures for lignocellulosic substrates degradation is of interest and open perspectives for development of optimized enzyme cocktails for degradation of biomass. *Trichoderma reesei* encodes a range of cellulases and hemicellulases for biomass degradation fungi and its enzymes are commonly used as industrial cellulase source,[10] however, *T. reesei* not is source of oxidoreductases enzymes.[11] Fungi of the genera Pestalotiopsis are recognized by

the production of oxidoreductases enzymes[12,13] and in this study was identified an Pestalotiopsis sp BBF245 strain producing oxidoreductases enzymes. The present study was developed in order to identify the hydrolytic efficiency of the enzymatic extract obtained from a newly-isolated strain of Pestalotiopsis sp BBF245, cultivated using sugarcane bagasse as substrate, and the *T. reesei* enzymatic extract in the sugarcane bagasse hydrolysis. The results show that the combination of lignocellulose-degrading enzymes significantly increased the degradation of sugarcane bagasse and suggest that the use of enzyme cocktails oxidoreductases and cellulases may significantly improve the hydrolysis of biomass.

Material and methods

Strains and culture conditions

The fungal strain was isolated from the air, collected from Bananal city, São Paulo state, Brazil, in April 2013. Cultured on slants of potato dextrose agar (PDA) at 28 °C for 4 days, the isolated strain was identified morphologically as Pestalotiopsis sp BBF245, which was further confirmed by ITS and LSU rDNA sequence, B-tubulin[14] and Elongation factor 1-alpha[15] DNA sequence analysis. *Trichoderma. reesei,* strain QM9414, was obtained from the American Type Culture Collection (ATCC 26921). A portion of fungus culture *Pestalotiopsis* sp in PDA medium was first cut into small pieces of 0.5 cm3 and shifted to a flask containing potato dextrose broth, and then incubated 30 ºC on a rotary shaker at 150 rpm for 7 days to obtain mycelial mass, that was recovered by centrifugation at 4000 rpm for 10 minutes and inoculated in Kirk culture medium[16] supplemented with 0.1% metal solution (0.5% FeSO4; 0.16% MnSO4; 0.16% ZnSO4 and 0.2% CaCl2), incubated at 30 °C, 150 rpm, for time determined by each experiment. When necessary, it has been added sugarcane bagasse in natura, from the plant Granelli LTDA (São Paulo, Brazil).

To obtain the crude supernatant of T.reesei, spore suspensions (107 spores/ml) was cultivated in medium PDB and grown for 5 days at 30 ° C and 150 rpm. The mycelial mass was centrifuged at 4000 rpm, recovered and inoculated into minimal medium[17] supplemented with 1 % Cellulose powder (Avicel PH-101, Sigma-Aldrich, USA). The culture was maintained at 30 °C with constant agitation and aeration. Aliquots of the culture were withdrawn to enzymatic activity of cellulases.

To obtain the crude supernatant of *Pestalotiopsis* sp. 1 cm^2 of the colony grown in PDA was inoculated in PDB and grown for 7 days. Afterwards the mycelial mass was centrifuged at 4000 rpm, recovered and inoculated in kirk medium containing sugarcane bagasse (0.5%) and supplemented with 100 µL of metal solution. On day 4 of culture the culture medium was supplemented with $CuSO_4$ 250 µM and veratryl alcohol 0.5 mM. Aliquots were removed from the culture medium and assays were performed to determine the enzymatic activity of cellulases and oxidoreductases. On the 8th day of culture the fungal pellets were removed by filtration, the supernatant was stored at -20°C and used in enzymatic activity assays to determine cellulase and oxidoreductases activity and in saccharification experiments on sugarcane bagasse.

Protein concentration was measured by the Bradford method, using the Quick Start Bradford Protein Assay Kit 1, and bovine serum albumin as standard (Bio-Rad Laboratories, USA).

Enzymatic assays

Endo glucanase and exo glucanase activities were assayed by the detection of reducing sugars from carboxy methyl cellulose 1% and avicel 1%, respectively. Reducing sugars were detected by the dinitrosalicylic acid (DNS) method[18], using glucose as standard. Briefly, activity was measured using 40µL of the substrate solution, 20µL of 0.5 M citrate buffer (pH 4.8) and 140µl of enzyme extract. Samples were incubated for 1 hour at 40 °C, being added 400 µL of DNS. The reaction was stopped by adding 400µL of H_2O. One unit (U) of enzymatic activity was defined as the amount of enzyme required to release 1µ mol of reducing sugar per minute, under assay conditions.

Laccase activity was determined using 2.2-azino-bisethylbenthiazolina (ABTS).[19] The mixture was composed by 60µL of 250mM sodium tartrate buffer (pH 4.2), 70µL of H_2O and 40µL enzyme solution. The mixture was incubated at 30 °C for 3 min and the reaction was started by adding 30µl of ABTS 5mM. The activity was monitored at 420 nm from the ABTS oxidation in ABTS+. MnP activity was measured by guaiacol oxidation method at 465 nm.[20] The reaction mixture was composed by 100µL of 250mM sodium tartrate buffer (pH 4.2), 2µL of 400mM guaiacol, 20µL of 10mM manganese sulfate and 70µL enzyme solution. The mixture was incubated at 30 ◦C for 3 min and the reaction was started by adding 10µL of 1mM H_2O_2. LiP activity was determined by the oxidation of veratryl alcohol.[16] The mixture reaction was composed by 100µL of 250mM sodium tartrate buffer (pH 4.2), 20µL of 100mM veratryl alcohol and 70µl of the enzyme extract. The reaction was incubated at 30 ºC for 3 min and the reaction was started by adding 10µL of 1mM H_2O_2 and the appearance of veratraldehyde was determined at 310 nm. One enzyme unit was defined as 1µMol of product formed per minute under the assay conditions. Activity of oxidoreductase enzymes was measured spectrophotometrically in the TECAN Infinite M200pro microplate reader. The reactions were performed in 96-well plates (SPL Life Sciences) in triplicate and the readings monitored in 10 cycles of 30 seconds.

Enzymatic saccharification of sugarcane bagasse

Enzymatic hydrolysis (EH) experiments were carried out using 100 mL flasks with 50mM citrate buffer at pH 5, in an incubator (HB-1000 Hybridyzer - UVP), with agitation speed of 100 rpm /50 ºC. The sugarcane bagasse (SB) was applied at a concentration of 4 % (w/v) of substrate total solids (TS). The working volume was 25.0 mL, and all experiments were performed in triplicate. Hydrolysis reactions were carried out in three assays: 1:0.75, 1:1 and 1:1 (5x concentrate) (v/v) supernatants proportion from *Trichoderma reesei* and Pestalotiopsis sp BBF245 respectively. Supernatant from the fungi was concentrated 5x using a crossflow cassette concentrator (Vivaflow 50, 10 kDa, Sartorius, USA). Supernatants were withdrawn after 0, 2, 4, 6 and 24 hours after incubation and the samples were then centrifuged 12,000 rpm / 10ºC for 15 min. The EH was measured by quantification of the reducing sugars released (with glucose as standard) according to the DNS method.[18] Control experiments were performed without supernatant addition of Pestalotiopsis sp BBF245 using only supernatant from *T. reesei.*

Results and discussion

Characterization of enzymatic extracts from fungi

The fungal strain Pestalotiopsis sp. BBF245 was isolated on PDA medium, identified morphologically and with based on partial LSU, ITS rDNA, B-tubulin and Elongation factor 1-alpha DNA sequence. Pestalotiopsis sp BBF245 belongs to the Xylariales Order, a group including a large number of soft-rot and lignolytic fungi that is still poorly explored and has shown great potential for the discovery of

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new lignocellulolytic enzymes.[13] Pestalotiopsis genus is the most commonly isolated endophytic fungi of tropical plants and has been shown to produce a wide range of biomolecules[21,22] to produce oxidative exoenzymes[23] and to overproduce laccase in solid-state fermentation of lignocellulosic by-products as substrates.[24] The initial characterization of the enzymatic extract from Pestalotiopsis sp BBF 245 growth in SCB was performed in terms of its production efficiency of oxidoreductases enzymes. The enzymatic extract shows high level of laccase activity (7.18 U/mg) and low activity of MnP, LigP (0.25 and 0.12 U/mg, respectively) and cellulases enzymes (Figure 1). As demonstrated by Rao et al.,[25] and Hao et al.,[12] enzyme activity of cellulases is low[25] and of laccase is high[12] in Pestalotiopsis strains. In this study we used the fungus *T. reesei,* one of the most well studied cellulolytic microorganisms and the major source for industrial cellulase[10] the production of enzymes in the third day was 7,0 and 2,1 U/mg of specific activity to endoglucanase and exoglucanase enzymes, respectively.

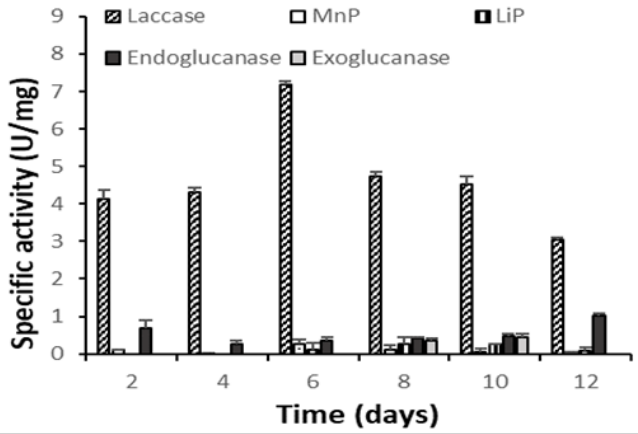

Figure 1 Specific activity of oxidorreductases and celulases of *Pestalotiopsis* sp.

Pestalotiopsis sp. extract supplementation with T. reesei extract

To determine the degradation of in natura SCB we analyzed the sugars that were released using DNS assay. In order to compare the efficiency of saccharification of sugarcane bagasse using fungal enzymes of *T. reesei* (control) and enzymatic extract of Pestalotiopsis sp, the enzymes mixture, saccharification assays were performed using in natura SCB (4% w/w – dry basis, at 50 ºC, for 24 h), in three different conditions (table 1), over a time course of 2, 4, 6 and 24 hours post-incubation (Figure 2). Table 1 presents the experimental conditions and the responses for the concentration of reducing sugars (g/L) after 24 h. Figure 2 shows the changes in the proportions of reducing sugars over the course of the experiments. We observed bagasse degradation after 2 h post-incubation, as shown in figure 2, mixture of enzymes showed a positive effect on EH efficiency. The amount of reducing sugars obtained during EH using the *T. reesei* supernatant (control) without oxidorreductases was 12.3 g/L (Figure 2). Enzyme cocktails with cellulases and oxidorreductases improved the amount of reducing sugars released by up to 25% (15.4 g/L was achieved during run 3). In the run 3, the specific activity (U/mg) of enzymes was 7.41; 0.05; 0.25; 5.94 and 1.57 to laccases, MnP, LiP, endoglucanases and exoglucanases, respectively.

The positive effect of cellulases and oxidorreductases on EH efficiency can be explained by the removal of lignin from the cell walls, which can further contribute to the action of cellulases. The changes in the proportion of sugars (Figure 2) led us to conclude that both cellulose and hemicellulose were being degraded. As mentioned before, the composition of sugarcane cell wall revealed that lignin content is of 4.9% in leaves and 2.5% in the culm.[26] These results indicate that proportions among enzymatic groups with the purpose of achieving higher hydrolysis yields can be related to substrate composition and that to increase hydrolysis yield of lignocellulosic biomass, more specific researches must be conducted, where the influence of lignin content be evaluated. Previous studies on the saccharification of others lignocellulosic biomass, using only cellullases enzymes shown that reducing sugar were obtained at a lower level (Corn stalk 5.2 g/L,[27] sawdust 2.88 g/L,[28] Sugarcane bagasse 10.19 g/L, [29] barley straw 3.8 g/L,[30]), however, Tuncer and Ball,[2] show that using cocktail enzymes containing peroxidase, endoglucanase, β-xylosidase, and α-L-arabinofuranosidase improve the hydrolysis of wheat straw.

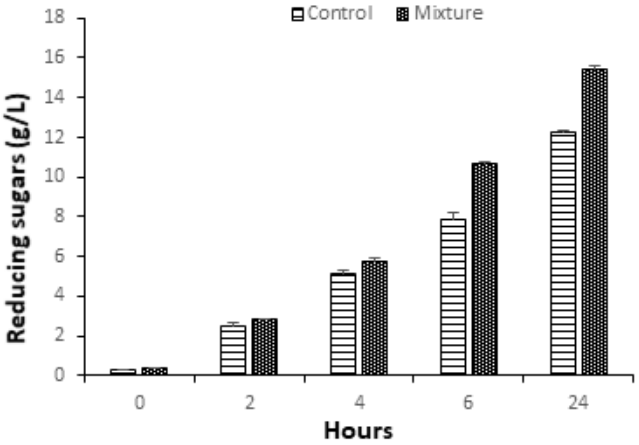

Figure 2 Reducing sugars released during saccharification of sugarcane bagasse in assay 3 (Table 1).

Table 1 Design for assay of different enzymatic cocktail mixture

Assay	Mixture (*T. reesei*: *Pestalotiospsis* sp.)	Reducing sugar (g/L) (only *T. reesei*)	Reducing sugar (g/L) (Mixture)
2	1:01	1,6	2,54
3	1:01	12,29	15,52

Conclusion

The reducing sugar produced by enzyme cocktails of cellulases or cellulases plus oxidorreductases were 12,29 and 15,52 g/L, respectively, the yield of reducing sugars produced through the degradation of SCB by enzyme cocktails suggests that these enzymes are capable of efficiently hydrolyzing the substrate. In this study we show that the enzymes of the cellulolytic and oxidoreductase complex obtained from *T. reesei* and Pestalotiopsis sp, were capable of hydrolyzing the SCB substrate, significantly increasing the reducing sugar production. The results highlight the role of oxidorreductases enzymes in the lignocellulose-degrading from SCB and suggest that the use of enzyme cocktails (cellulase plus oxidorreductases) may significantly improve the hydrolysis of SCB in industrial processes. The results obtained demonstrate that fungi Pestalotiopsis sp has bio technological potential to be used in bioprocesses that aim at obtaining enzymes for later use for bioconversion of cellulose into glucose and of the industrially important enzyme laccase.[12] The low levels of cellulolytic activity exhibited by Pestalotiopsis sp makes it potentially suitable for use in municipal wastewater treatment plant where laccase enzyme appear to be a promising biocatalyst to enhance the

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biodegradation of micro pollutants in wastewater in a complementary treatment step.[31] These results suggest that the cellullases and oxidorreductases act synergistically on the lignocellulose polymer and are capable of releasing reducing sugars from the substrate and that a significant increase in degradation can be achieved with the cooperative actions of lignocellulose-degrading enzymes from *T. reesei* and Pestalotiopsis sp BBF245. This enhanced activity using synergism between enzymes confirms a potentially wider role for the enzyme combinations in industrial applications,[32–34] suggesting that more efficient hydrolysis of lignocellulose requires the interactions of more lignocellulose-degrading enzymes. Finally, since any biotechnological process to lignocellulose-degrading is likely to be based on crude extracts of enzyme cocktails, it is important to increase any particular enzymes activity in culture supernatants to improve the hydrolysis of substrate in the industrial processes.[6]

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None

Conflicts of interest

No conflicts of interest.

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