A NOVEL *Scytalidium* SPECIES: UNDERSTAND THE CELLULOLYTIC SYSTEM FOR BIOMASS SACCHARIFICATION

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Abstract - In order to overcome the bottlenecks related to lignocellulosic-derived sugars, the search for more efficient enzymatic cocktails, containing a broad-spectrum of specific activities, relies on an important feature. This paper describes new enzyme activities derived from the novel strain of the *Scytalidium* genus isolated from the Amazonas rainforest. The production of the enzymatic cocktail was induced by delignified-hydrothermal bagasse (DHB), and yeast extract was used to improve secretion activities, resulting in a positive influence on total cellulase activity. The enzymatic cocktail produced by this novel strain contains specific activities for biomass degradation, including FPAse, xylanase and β-glucosidase. Moreover, it is capable of hydrolyzing 62% of the alkaline pretreated bagasse, surpassing in 14% the hydrolytic capability achieved by the commercial cocktail Celluclast. To this extent, the strain described here emerges as a reliable alternative to other available enzymes and, consequently, amplification of available specific substrate activities.
Keywords: *Scytalidium* sp; Cellulases; Sugarcane bagasse; Saccharification.

INTRODUCTION

Many enzymes are required for the efficient hydrolysis of lignocellulosic materials. Cellulases (endoglucanases, exoglucanases, and β-glucosidases) and xylanases are the main enzymes involved in lignocellulose deconstruction, and they act in synergism with accessory enzymes, such as hemicellulolytic enzymes (Goldbeck et al., 2016), feruloyl esterase (Braga et al., 2014), pectinases and a-L-arabinofuranosidase (Delabona et al., 2012). However, the high costs associated with these enzymes, which vary from $0.10/gal to $0.40/gal, and the bottlenecks related to feedstock and pretreatment make enzymatic hydrolysis unfeasible for large-scale 2G ethanol production (Johnson, 2016).

New fungi strains and consequently new enzymes can be the key for a more profitable biomass degradation, especially when considering the wide range of substrate specificities and the improved biophysical properties. Thereby, hydrolases of microorganisms from diverse environments such as desert (Moreno et al., 2012), marine habitat (Padmavathi et al., 2012), rain forest soils (Delabona et al., 2012) and plants (Robl et al., 2015) have been extensively explored. In this respect, the Amazon biodiversity is considered to be an open field to search for microorganisms that secrete powerful enzymatic cocktails (Delabona et al., 2012).

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Scytalidium is generally classified as a dematiaceous (dark-walled) fungal genus, known for hosting by several plants. The genus contains about 18 species and is characterized by the presence of intercalary or terminal arthroconidia and chlamydospores (Kirk et al., 2008). There are some species that can cause onychomycosis in tea pluckers (Barua et al., 2007). However, there are others with biotechnological applications such as *S. thermophilum*, that has been considered as a potential candidate in the biostoning of denim garments, since appreciable amounts of cellulases are produced along with xylanases (Kaur et al., 2006). Another potential species of *Scytalidium* is *S. acidophilum,* which grows under extremely acidic and high-temperature conditions, being able to secrete enzymes for processes that require extreme low pH's and/or high temperature (Balaa et al., 2009).

The carbon source used in cultivations is one of the most important factors affecting the cost and yield of enzymatic mixture production. The aim of this study was to explore the capacity of a novel *Scytalidium* filamentous fungus to produce biomass-conversion enzymes using different lignocellulosic substrates. The enzymes produced by the new strain using optimized seeding broth were proposed as a novel enzymatic cocktail for biomass saccharification compared with commercial enzyme preparation.

MATERIAL AND METHODS

Strain

The strain of *Scytalidium* sp. MIBA247 used in the present study was isolated from the Amazon Forest and provided by Dr. Alberdan Silva Santos (Biotechnology Systematic Research and Molecular Biology Laboratory, LabSisBio, Federal University of Pará, Pará, Brazil). A total of 156 Brazilian filamentous fungi were selected, previously isolated from Amazon soil. The strains were stored on potato dextrose agar (PDA) slants at 4°C.

Plate screening and fungal identification

The 156 isolates were initially screened based on their ability to grow on a synthetic medium containing Avicel (Fluka Biochemika, Switzerland) as the sole carbon source. The strains were first grown on PDA for 7 days at 29°C, and then inoculated into the test media and incubated for 48 h at 29°C. The composition of the test media was as follows: $NaNO_3$ (3.0 g/L); K_2HPO_4 (1.0 g/L); $MgSO_4$ (0.5 g/L); KCl (0.5 g/L); $FeSO_4 \cdot 7H_2O$ (0.01 mg/L); agar (20.0 g/L); Avicel (5.0 g/L). The pH was adjusted to 5.0 prior to sterilization. Inoculation was carried out by using a platinum needle to transfer the spores from the PDA plate to the center of the plates containing the Avicel medium. The inoculated plates were incubated for 48 h at 29°C and the growth

of the microorganism was measured by the diameter of the colony. A 10 mL aliquot of Congo red dye (2.5 g/L) was then added to each plate. After 15 min, the solution was discarded and the cultures were washed with 10 mL of 1 mol/L NaCl. Cellulase production was indicated by the appearance of a pale halo with orange edges, indicative of areas of hydrolysis. This halo was measured for subsequent calculation of the enzymatic index (EI), using the expression:

$$EI = \frac{\text{diameter of hydrolysis zone}}{\text{diameter of colony}} \quad (1)$$

The strains that showed an EI higher than 0.5 were considered to be potential producers of cellulases and the best strain was selected for identification. The fungal DNA from the best isolate was extracted using Qiagen Dneasy plant mini kit, following the manufacturer's instructions. The isolated DNA was used for the amplification of the internal transcribed spacer regions (ITS) by PCR reaction using the universal primers ITS1 (Forward primer) and ITS4 (Reverse primer) (White et al., 1990). The complete sequences representing the ITS1 and ITS2 regions were compared against the Fungal Barcoding Database (http://www.fungalbarcoding.org/). This database is composed of Q-bank Fungi database (http://www.q-bank.edu/Fungi), CBS-KNAW Fungal Biodiversity Centre (http://www.cbs.knaw.nl/Collections), Molecular Mycology Research Laboratory (http://www.mycologylab.org/), Portuguese Yeast Culture Collection (http://pycc.bioaware.com/) and the National Center for Biotechnology Information (NCBI). For the phylogenetic relationships analysis, the complete ITS sequence from MIBA 0247 isolate was compared with ITS sequences from others known and characterized fungi. The ClustalX 1.83 software was used to align the ITS sequences (Thompson et al., 1997). The phylogenetic tree was constructed with the aid of Mega 4 program (Tamura et al., 2007) using the nearest neighbor algorithm "neighbor-joining" (Saitou and Nei, 1987) with the nucleotide substitution model 2P (Kimura, 1980). The statistical bootstrap method was used to simulate and evaluate the reliability of the construction of the phylogenetic tree (Efron and Tibshirani, 1994). The ITS sequence obtained in this study was registered in National Center for Biotechnology Information (NCBI) under the accession number KT877403. All the strains isolated in the present work are kept at the Biotechnology Systematic Research and Molecular Biology Laboratory (Belém, Brazil).

Shake flask fermentation

After the screening of the cellulolytic potential of the fungi, the strain that showed an EI higher than 0.5

was selected for quantitative evaluation using shake flask fermentation. The strain was grown on PDA plates (90 x 15 mm) at 29 °C for 7 days. A conidia suspension was prepared by adding 20 mL of sterilized distilled water and Tween 80 to the grown PDA plates. This suspension was transferred to Erlenmeyer flasks containing 180 mL of pre-culture medium adapted from Mandels and Weber, 1969. The composition of the medium was as follows: 1 mL Tween 80; 0.3 g L^{-1} urea; 2.0 g L^{-1} KH_2PO_4; 1.4 g L^{-1} $(NH_4)_2SO_4$; 0.4 g L^{-1} $CaCl_2.2H_2O$; 0.3 g L^{-1} $MgSO_4.7H_2O$; 1.0 g L^{-1} proteose peptone; 5.0 mg L^{-1} $FeSO_4.7H_2O$; 1.6 mg L^{-1} $MnSO_4.4H_2O$; 1.4 mg L^{-1} $ZnSO_4.7H_2O$; 2.0 mg L^{-1} $CoCl_2.6H_2O$; 10 g L^{-1} glucose as carbon source. The induction medium was prepared with the same pre-culture medium (without glucose) supplemented with 10 g/L of different carbon sources (Table 1). The pH of the culture medium was adjusted to 5.0 before sterilization in an autoclave at 121 °C for 20 min. The assays were performed in duplicate, with incubation of the shaker flasks for 120 hours at 29 °C and 200 rpm in an Innova incubator (Eppendorf, USA). Samples were periodically withdrawn, centrifuged at 10,000 g, 10 °C for 15 min and analyzed for protein content and enzymatic activities.

Optimal media composition design

The induction media containing delignified-hydrothermal bagasse (DHB) was supplemented with sucrose (SUC) and yeast extract (YE) and its composition was studied in order to maximize filter paper activity (FPase). Optimization of DHB, SUC and YE concentration was assessed using a Central Composite Rotational Design (CCRD). The CCRD (Table 2) was performed by testing different levels of DHB (10-20 g L^{-1}), SUC (1.55-6 g L^{-1}) and YE (1-3 g L^{-1}) at initial pH 4.7 using 0.1 M biphthalate buffer to minimize pH fluctuation (Dos Santos Costa et al., 2016). The backward elimination technique was applied to ensure maximization of the regression coefficient of the proposed mathematical model describing the relationship between the FPase enzymatic activity and the DHB, YE and SUC concentrations. To validate the mathematical model, analysis of variance (ANOVA) was performed. All statistical treatments were carried out with the aid of Minitab® 17 (Minitab Inc., USA).

Bioreactor fermentation

The experiments were carried out in a Bioflo 115 bioreactor (New Brunswick Scientific, CT, USA) with a working volume of 1.5 L, at 29 °C and pH 5.0 (controlled using 0.4 M H_2SO_4 and aqueous NH_4OH solution, in a 1:4 ratio), and with a dissolved oxygen concentration above 30% of air saturation. Foaming was controlled by using polypropylene glycol antifoaming agent (P2000, Dow Chemical, Brazil), at an initial concentration of 1 mL/L. The inoculum was performed by transferring 10% (v/v) of the pre-culture to a bioreactor. The medium which presented the maximized response in shake flasks was validated in a bioreactor assay. The effect of incrementing the component concentrations (DHB, SUC and YE) was also investigated by performing cultivations using 10 g/L and 30g/L of carbon source. Then it was possible to understand the effect of carbon source concentration on enzyme activity (FPase, xylanase, and β-glucosidase) and total protein over time. Samples were periodically withdrawn, centrifuged at 10,000g for 20 min (at 10 °C) and measured in relation to enzymatic activities, as well as protein content, as described below.

Enzymatic assays and total protein determination

Quantification of enzymatic activities in the supernatants, expressed as international units (IU), was performed after cultivations using different substrates in order to determine global and single activities. Filter paper activity (FPase) was determined as described by Ghose (1987), with modifications to diminish the scale of the procedure by a factor of 10. As sugar yield and the quantity of enzyme in the assay are not correlated by a linear function, FPU should be determined only at the defined enzyme dilution needed for the liberation of 2 mg of glucose. In order to calculate the enzyme dilution needed to release 0.2 mg of glucose (due to the reduced scale of the procedure by a factor of 10), at least two dilutions should be prepared, with one dilution releasing slightly more than 0.2 mg of glucose and the other slightly less than 0.2 mg of glucose. The substrate for this reaction is 5 mg Whatman No. 1 filter paper. The enzyme assay tube is prepared by the addition of the substrate, 100 μL 0.05 M citrate buffer pH 4.8 and 50 μL enzyme preparation. The experimental tubes are incubated at 50ºC for 60 min in a water bath. The color development and sugar quantification were carried

Table 1. Composition of the carbon source for cellulase production *screening* in submerged flask fermentation.

Carbon source	Cellulose	Hemicellulose	Lignin	Ash
	(%)			
Celufloc 200™ (CE)	86.01 ± 0.49	14.91 ± 0.22	0.97 ± 0.08	0.15 ± 0.04
Steam-pretreated Bagasse (SB)	57.1 ± 0.3	10.9 ± 0.1	27.6 ± 0.8	4.6 ± 0.0
Delignified Steam-exploded pretreated Bagasse (DSB)	74.0 ± 0.1	6.8 ± 0.1	16.2 ± 0.0	4.9 ± 0.1
Hydrothermal pretreated Bagasse (HB)	74.0 ± 1.3	6.0 ± 0.5	26.0 ± 0.1	0.2 ± 0.1
Delignified Hydrothermal pretreated Bagasse (DHB)	83.4 ± 0.2	2.2 ± 0.1	26.0 ± 0.1	5.8 ± 0.8
Alkaline pretreatment Bagasse (AB)	63.1 ± 3.8	21.1 ± 1.5	7.1 ± 2.0	8.3 ± 2.7

out according to the method proposed by Miller (1959). After the identification of two data points that release very close to 0.2 mg of glucose, the enzyme dilution that produces exactly 0.2 mg of glucose can be calculated by plotting glucose liberated against the logarithm of enzyme concentration.

The calculation of the FPU follows the equation:

$$\text{Filter paper activity}\left(\frac{\text{units}}{\text{L}}\right) = \left(\frac{0.37}{[\text{enzyme}]\,\text{releasing 0.2 mg glucose}}\right)\cdot 1000 \quad (2)$$

where [enzyme] is the proportion of the original enzyme solution in the dilution, while 0.37 takes into consideration the conversion of 0.2 mg of glucose to mmoles of glucose, divided by the incubation time (60 min) and the volume of enzyme dilution being tested (0.05 mL).

All the polysaccharides used to measure the single activities were purchased from Sigma-Aldrich or Megazyme, and were assayed at 0.5% in a 10 min reaction (Delabona et al., 2013). The polysaccharides used were: Linear arabinan; Larch arabinogalactan; Icelandic moss lichenan; Tamarind xyloglucan; Wheat arabinoxylan; Konjac glucomannan; Chitosan from shrimp shells; Citrus pectin; Barley β-glucan and Beechwood xylan. Avicel and Carboxymethylcellulose (CMC) were assayed in a 30 min reaction. The enzymatic activity was determined from the amount of reducing sugars released from the different polysaccharide substrates, using the dinitrosalicylic acid (DNS) method (Miller, 1959) with glucose as standard. The activities of β-glucosidase, β-xylosidase, β-mannosidase, α-L-arabinofuranosidase, and cellobiohydrolase II were measured using the respective p-nitrophenol (pNP) residues (Sigma-Aldrich, USA). The assays employed 10 μL of diluted centrifugated supernatant and 90 μL of the respective pNP (0.5 mM, diluted in citrate buffer), and the mixtures were incubated for 10 min at 50°C. The reactions were stopped by adding 100 μL of 1M Na_2CO_3, and the absorbance was measured at 400 nm using a Tecan Infinite® 200 instrument (Männedorf, Switzerland). All the assays were performed at pH 5.0 with 50 mM citrate buffer. One unit of glycohydrolases activity corresponds to 1 μmol of glucose or pNP released per minute.

Total protein was measured in microplates using Bio-Rad protein assay reagent (Bio-Rad Laboratories, USA), using a procedure based on the Bradford method (Bradford, 1976). Bovine serum albumin was used as a standard.

Enzymatic hydrolysis (HE)

The alkaline pretreated bagasse (BA) was prepared and characterized according to Nascimento et al. (2016) and subjected to enzymatic saccharification using an enzymatic cocktail from *Scytalidium* sp. MIBA247. The enzymatic cocktail was obtained by cultivation of the referred strain in a bioreactor using optimized media as substrate (DHB = 10.83 g/L and YE = 3.68 g/L), resulting in 1300 FPU/L of cellulase, 163500 IU/L of xylanase, and 122 IU/L of β-glucosidase. A comparison of substrate conversion was made with the enzyme preparation Celluclast® 1.5L, which contained 86050 FPU/L, 429300 IU/L of xylanase and 89610 IU/L of β-glucosidase. Enzymatic hydrolysis experiments were carried out using 125 mL Erlenmeyer flasks containing 50 mM citrate buffer at pH 4.8, in an Innova incubator (Eppendorf, USA) operated at an agitation speed of 250 rpm and temperature of 50 °C. The BA was applied at a concentration of 10% (w/v) of substrate total solids. For both enzymatic cocktails, the enzyme load was equivalent to 10 FPU per gram of pretreated biomass. The working volume was 50 mL, and all experiments were performed in triplicate. Samples were periodically withdrawn (0.5, 4, 10, 24, 48 and 72 hours) and centrifuged at 10000g for 10 min at 4°C. The clarified supernatant was incubated for 5 min at 95°C and frozen for posterior analysis. Glucose, cellobiose, and xylose were separated with an Aminex HPX 87H (300 × 7.8 mm, BIO-RAD) at 45 °C using 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min, and detected with an infrared (IR) detector (Shimadzu RID-6A).

RESULTS AND DISCUSSION

Screening and ITS identification

The MIBA 0247 strain showed the best potential for Avicel degradation and then was selected for shake flasks cultivation. Initial screening of the 156 isolates was carried out by assessment of fungal growth on plates containing Avicel microcrystalline cellulose as the sole carbon source. Congo Red stain revealed the hydrolysis halos, which was used for calculation of the enzymatic index. According to Ruegger and Tauk-tornisielo (2004), the enzymatic index can be used as a simple and rapid methodology to select strains. The strains that showed an EI higher than 0.50 were considered to be potential producers of cellulases. A total of 30 strains were unable to grow on microcrystalline cellulose, while only 21 strains as well as the reference strain (*T. reesei* RUT C30) exhibited considerable growth and produced halos with EI ≥ 0.5. The halo produced by hydrolysis of cellulose is directly related to the site of cellulolytic enzyme action and this screening performed on agar plates has been widely used. Florencio et al. (2012) studied the correlation between agar plate screening and solid-state fermentation for the prediction of cellulase production by *Trichoderma* strains. The authors demonstrated a direct relationship between

the quantitative and qualitative tests since the strains with higher EI showed higher production of cellulases. Therefore, these methods in plates are feasible as they can be employed for a pre-selection of strains, let alone the fact of being simple, rapid and well adapted for screening a large number of samples.

The molecular identification of the fungal isolate MIBA 0247 was performed by pairwise sequence alignment from the ITS barcode against the fungal database. Due to their great variability, the internal transcribed spacer regions (ITS) have been a robust universal molecular marker widely used in fungal identification (Kiss, 2012). The amplicon obtained by PCR amplification using universal primers (ITS1 and ITS2 region) exhibited fragments with unique sizes of 562 bp. According to the results, the closest genus that matched the sequence from MIBA 0247 were representatives of the *Scytalidium sp*. However, the nucleotide sequence analysis of the ITS from MIBA 0247 resulted in a low similarity (87.5%) with previously described fungal ITS sequences. Usually, a cut-off level of 97% identity has been used to define species in studies involving fungal taxonomy (Nilsson et al., 2008). In addition, a neighbor-joining phylogenetic tree containing several NCBI database sequences was created to analyze the phylogenetic relationships among different species of *Scytalidium sp*. All the sequences for ITS together with their GenBank accession numbers used in the alignments are presented in the phylogenetic tree (Fig. 1). As expected, we observed that most organisms were grouped into distinct clades according to their respective species.
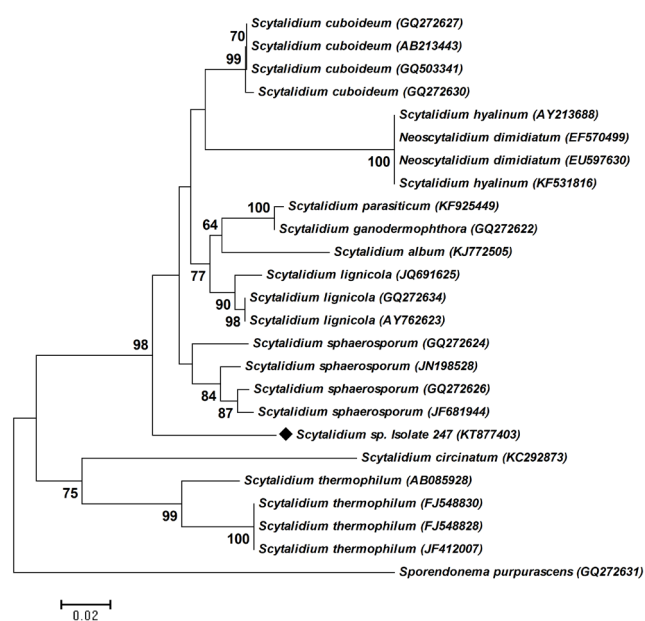

Figure 1. Phylogenetic dendrogram based on the sequences of ITS rDNA regions indexed in the National Center for Biotechnology Information (NCBI) database, grouped by species.

However, interestingly MIBA 0247 did not share a clade with any of the representative members from known strains of *Scytalidium sp*. The phylogenetic tree analysis also showed clearly that MIBA 0247 was separated by an external branch from previously described *Scytalidium* species. The reliability of the branch was strongly supported by 98% Bootstrap values. Based on the results described above, we suggest that our isolate is significantly different from known *Scytalidium* species in the literature. Thus, we can propose that the MIBA 0247 isolate identified in this paper belongs to a new species of the *Scytalidium sp*. genus.

Effect of carbon source on enzyme production in shake flasks

This study explored the inherent capacity of the novel *Scytalidium* filamentous fungus to produce biomass-conversion enzymes upon growth on lignocellulosic substrates. The amounts of secreted protein and the activities of the cellulolytic enzymes were influenced by the type of cellulosic material (Fig. 2). Five different pretreated lignocellulosic substrates derived from sugar cane bagasse (steam-pretreated bagasse (SB), delignified hydrothermal pretreated bagasse (DSB), hydrothermal pretreated bagasse (HB), delignified hydrothermal pretreated bagasse (DHB) and alkaline pretreated bagasse (AB)) and commercial cellulose (CE) were used for fungal growth in shake flasks. We aimed to disclose a wide range of lignocellulose degrading capacity associated with the preselected strain, revealed in terms of high FPAse, xylanase and β-glucosidase activity, due to the use of a diverse set of substrates for fungus cultivation.

The greatest enzymatic activity was registered when the microorganism was cultivated in DHB, where the maximum FPase activity recorded was 616.58 ± 0.02 U/L at 96 h of fermentation. The enzymatic activity released under this condition was followed by cultivation in CE and DSB, with maximum FPase activity of 594.09 ± 0.01 U/L and 575.13 ± 0.01 U/L, respectively. Steam-pretreated bagasse (SB) presented the lowest enzymatic production, with maximum filter paper activity less than 400 FPU/L.

As far as xylanase activities were concerned, the maximum values were recorded at 72 h of fermentation, being higher when the strain is cultivated with DHB (± 66.000 IU/L), followed by DSB (± 61.000 IU/L) and CE (± (56.000 IU/L) (Fig. 2B). Among the enzymatic activities analyzed, β-glucosidase measurement resulted in the smallest quantities (Fig. 2C). The maximum β-glucosidase activity was recorded after 120 h cultivation. The best results were obtained using commercial microcrystalline cellulose (CE) as a substrate, followed by fermentation employing DSB, wherein at 120 h fermentation β-glucosidase activities
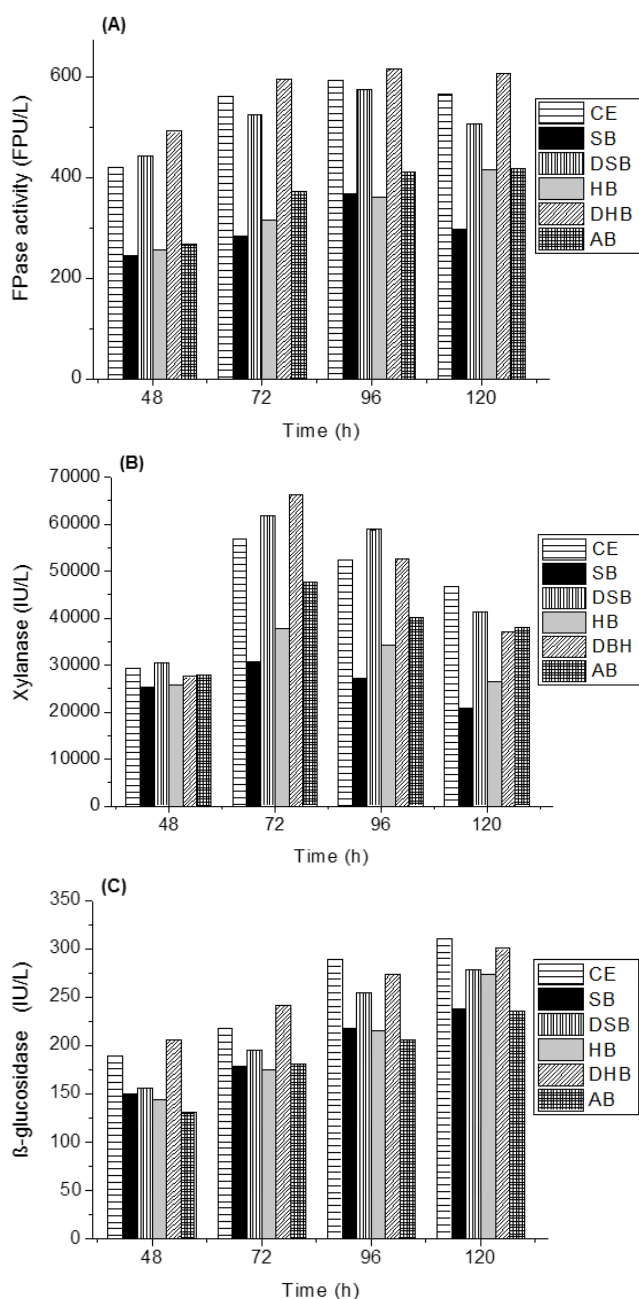
(CE - commercial cellulose; SB - steam-pretreated bagasse; DSB - delignified hydrothermal pretreated bagasse; HB - hydrothermal pretreated bagasse; DHB - delignified hydrothermal pretreated bagasse; AB - alkaline-pretreated bagasse).

Figure 2. Influence of different carbon sources on the FPase (A), xylanase (B) and β-glucosidase (C) production by *Scytalidium* sp. during submerged fermentation in flasks.

were 310.49 U/L and 300.47 U/L, respectively. Because microcrystalline cellulose is a substrate with predominant crystalline structure, it may likely induce the production of cellobiohydrolases, whose product is cellobiose, a substrate easily hydrolyzed by β-glucosidases in only one step (Zhang et al., 2007). According to Maeda et al. (2011) and Goldbeck et al. (2014), a natural microorganism capable of

producing an optimal enzyme composition needed for degradation of lignocellulosic biomass has not yet been encountered. Therefore, if a certain microorganism presents a lack of a specific enzyme activity, this activity can be supplemented with native or recombinant enzymes for use in particular biotechnology applications (Delabona et al., 2013).

Therefore, the results demonstrated that the type of biomass pretreatment influences the amounts and types of cellulolytic enzymes secreted during the strain's growth. Cultivation on sugarcane bagasse submitted to a delignification process resulted in the highest cellulolytic activities. The delignification enables the removal of lignin and consequently the reduction of negative effects associated with toxic compounds. Based on these results, DHB was selected as a substrate to be used in the optimization of cellulase production studies in the bioreactor.

Optimal media composition design

Culture media optimization based on the design of experiments approach has been successfully used to increase cellulase production (Delabona et al., 2013; Dos Santos Costa et al., 2016). In the present study, the culture medium was composed based on our previous work (Delabona et al., 2016) using DHB as the main carbon and inductor source. The medium was supplemented with yeast extract and sucrose, both inductors for cellulase and β-glucosidase production during *Trichoderma harzianum* (Delabona et al., 2012) and *Penicillium echinulatum* (Dos Reis et al., 2015; Dos Santos Costa et al., 2016) culture.

A central composite rotational design of experiment with two-levels and 3 factors (Table 2) was conducted to infer the influence of the culture medium components pretreated sugarcane bagasse (DHB), yeast extract (YE) and sucrose (SUC) on the FPase enzymatic activity measured at 72h of shake flask *Scytalidium* MIBA 247 cultivation. The results of measured FPase activity for the different runs are presented in Table 2.

The analysis of variance of the data was done using the Backward Elimination Technique of Minitab 17 software.

The mathematical model below describes the relationship between measured FPase and the concentrations of the dependent variables.

$$FPase = 0.209 + 0.04168[DHB] + 0.0661[YE] - 0.001927[DHB]^2 \quad (3)$$

The equation coefficients are all significant at the 95 % confidence level. The hypothesis of lack-of-fit of the equation was rejected, as the p-value = 0.014 is lower than 0.05. The model passed on the F test as the F-value = 25.58 (Table 1, SM) is higher than $F_{5\%,3,14}$ = 3.34, meaning that the data was well correlated by the proposed model.

Table 2. FPase activity (FPU/L) of culture broth of shake flask *Scytalidium* sp. MIBA247 cultivation in culture media composed of delignified hydrothermal pretreated sugarcane bagasse (DHB); yeast extract (YE) and sucrose (SUC) at different concentration in g/L.

Assay	DHB	YE	SUC	FPase
		(g/L)		(FPU/L)
1	20.00	1.00	6.00	290
2	15.00	2.00	3.78	510
3	10.00	3.00	6.00	710
4	20.00	3.00	1.55	440
5	15.00	2.00	3.78	560
6	15.00	2.00	3.78	650
7	10.00	1.00	6.00	680
8	15.00	3.68	3.78	710
9	10.00	3.00	1.55	610
10	15.00	0.38	3.78	430
11	15.00	2.00	3.78	650
12	6.59	2.00	3.78	490
13	10.00	1.00	1.55	560
14	20.00	3.00	6.00	470
15	23.41	2.00	3.78	290
16	15.00	2.00	0.03	300
17	20.00	1.00	1.55	310
18	15.00	2.00	7.52	580

The response surface analysis using Minitab 17 was utilized to design a culture medium composition that maximized FPase enzymatic activity. As shown in Equation 2, only DHB and yeast extract influence FPase activity, in the range of concentrations studied. The increase in DHB concentration affected negatively FPase after approximately 10 to 12 g/L. On the other hand, the increase of YE benefited FPase response in the tested interval (1-3 g/L) (Fig. 3).

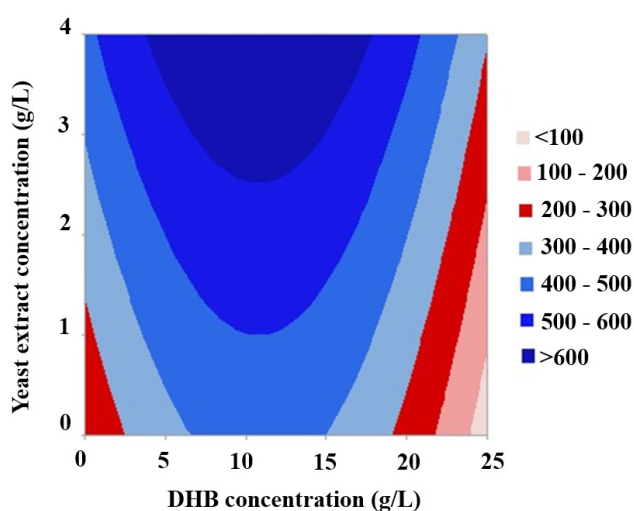Hence, the medium composition was optimized using the Response Optmizer of Minitab 17 by varying

Figure 3. Contour plots of FPase response for the *Scytalidium* sp. using the culture medium components (g/L) delignified steam-exploded bagasse (DHB) and yeast extract (YE) grown in submerged cultivation.

DSB and YE between its lowest and highest tested values as presented in Table 2, respectively, 6.59 to 23.41 g/L and 0.32 to 3.68 g/L, using same weight and importance (attributing for both parameters values = 1).

The optimized culture medium presented a composition of 10.83 g/L for DHB and 3.68 g/L for yeast extract, resulting in a maximized response for FPase of 688 FPU/L. This is in accordance with a surface contour plot of FPase versus tested components, yeast extract (YE) and delignified hydrothermal sugarcane bagasse (DHB) concentrations (Fig. 3).

Enzymatic activity profile by bioreactor fermentation

To validate the culture media composition, two batch experiments were carried out in lab scale (3 L) bioreactors at different concentrations of DHB (10 g/L and 30 g/L) and the same YE concentration of 3.7 g/L. A possible way to increase productivity and enzymatic activity is by using a higher initial concentration of carbon source, which is assumed to be the limiting substrate, in order to identify any positive effects on glycoside hydrolase activity. Figure 4A displays the FPase activity evolution over time. Maximum attained activity was 650 FPU/L at 72h fermentation for the experiment using 10 g/L of DHB. When the initial DHB concentration was set at 30 g/L, enzyme production decreased in the first 96 h and maximum FPase activity did not exceed 400 FPU/L. Interestingly, β-glucosidase and xylanase activities were also strongly negatively affected by increasing DHB concentration from 10 to 30 g/L. This similar profile for both activities suggests that these enzymes are coordinately produced in *Scytalidium* sp.

The influence of cellulose concentration on enzymes production has already been assessed using many filamentous fungi species, mainly *Trichoderma reesei,* that is the most widely used for the industrial production of cellulolytic and hemicellulolytic enzymes. An initial cellulose concentration up to 3% benefited cellulase production in *T. reesei* RUT C30, while a higher concentration decreased the enzyme production rate (Hendy et al., 1982). Similarly, Delabona et al. (2013) showed that the final titer of glycosyl hydrolases (GH) also increased for cellulose concentration up to 3% (w/v), but jeopardized the GH titer above this value for *Trichoderma harzianum* P49P11. The rise in the concentration of insoluble substrate in the bioreactor could hinder the mass transfer of substrates to the growing mycelial biomass, as well as the removal of inhibitory products.

Production of cellulase in cellulolytic fungi is regulated at the transcription level (Kubicek, 2012). Their synthesis is induced by the presence of cellulose in the culture medium, but it is repressed above a certain
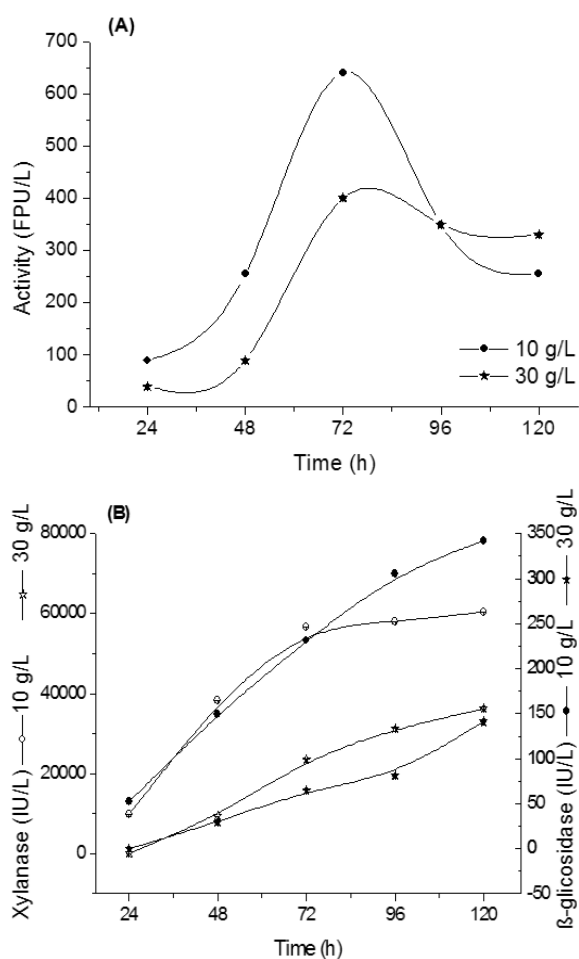
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Figure 4. *Scytalidium* sp. enzymatic activity profile in the bioreactor using DHB and YE culture media with initial concentrations (w/v) of 10 (circle) and 30 g/L (star): FPase (A), xylanase and β-glucosidase (B).

level of available glucose or other readily assimilable sugars in the fermentation broth by a mechanism named carbon (glucose) catabolite repression (CCR) (Ilmen et al., 1997). The role of glucose, cellobiose, sophorose and other soluble sugars as repressors or inducers of cellulase production varies with the microorganisms (Nakari-Setala and Penttila, 1995). The decrease of cellulase enzyme production with an increment of cellulosic substrate source has been described for several fungi and the CCR is a common control mechanism in filamentous fungi during cellulase biosynthesis (Suto and Tomita, 2001). The results indicate that a glucose repression mechanism remains in this filamentous fungus, hindering the use of high initial carbon/inductor concentration. Therefore, the experiments run in the bioreactor validated the optimized culture medium as proposed by Equation 2.

Glycohydrolase profile of enzymatic extracts

In order to identify different enzyme profiles and understand the cellulolytic system of a novel *Scytalidium* sp., a "panel of enzymatic activity"

was performed to characterize the activity profiles of the supernatants produced under bioreactor submerged fermentation using 10 g/L of DHB. This characterization was made using a wide selection of substrates, including ρ-nitrophenol (ρ-NP) derivatives and cellulosic and non-cellulosic materials (Table 3). The understanding of the enzymatic profile of novel strain regarding the degradation of sugarcane bagasse and the identification of enzymes that are lacking can contribute to a higher saccharification capacity in supplementation studies (Delabona et al., 2013).

Efficient degradation of cellulose requires the cooperation of at least three types of enzymes: cellobiohydrolases, endoglucanases, and β-glucosidases. Hemicellulose is degraded by endo-xylanase, β-xylosidase and debranching enzymes, such as α-arabinofuranosidase. Pectinases are a heterogeneous group of related enzymes that hydrolyze the pectin substances typically present in plants.

Pretreatment technologies such as steam explosion may extract some of the most soluble polymers. However, as mentioned before, a sub-population of pectin and hemicellulose fraction remains attached to the solid pretreated material (de Souza et al., 2013). Because of this, it is important to take into account the degradation of hemicellulose and pectin when looking for cellulose conversion, even though these polymers are not present in predominant amounts in the pretreated biomass. The action of xylanases, pectinase and other accessory enzymes can improve cellulose conversion by removing hemicellulose and increasing the accessibility of the substrate to cellulases.

The novel strain is able to produce the cellulolytic complex composed of the three essential classes of

Table 3. Glycohydrolase specific activities (IU/mg) of *Scytalidium* sp. growth using DHB and YE as carbon source.

Cellulosic Substrates	IU/mg
Avicel	nd
CMC	1.13
Filter paper *Whatman No. 1*	1.46
Non-cellulosic substrates	
Linear arabinan	0.71
Larch arabinogalactan	0.46
Lichenan	0.90
Xyloglucan	2.22
Wheat arabinoxylan	0.81
Glucomannan	2.20
Chitosan	0.88
Pectin	0.73
β-glucan	17.53
Beechwood xylan	371.11
ρ-Nitrophenol substrates	
pNP -D-glucopyranoside	0.52
pNP -L-arabinofuranoside	0.1
pNP -D-cellobioside	0.77
pNP -D-mannopyranoside	0.34
pNP -D-xylopyranoside	0.28

enzymes, translated into the following activities: 1.46 IU/mg of FPase; 1.13 IU/mg of CMCase and 0.52 IU/mg of β-glucosidase. Studies with *Scytalidium thermophilum* showed that it produces cellulases that are not inhibited by either glucose or cellobiose, which is a very interesting property for industrial biomass saccharification (Carlos et al., 2013). The supplementation of home-made cocktails with endoglucanase and β-glucosidase activities has already been reported to improve the saccharification of pretreated sugarcane bagasse, increasing the conversion of cellulose by approximately 40% compared to Celluclast 1.5 L, in hydrolysis experiments under the same conditions (Bussamra et al., 2015). The strain of *Scytalidium* studied here also showed high xylanase activity against xylan beechwood (371.11 U/mg), followed by β-glucan (17.53 U/mg), xyloglucan (2.22 U/mg) and glucomannan (2.20 U/mg). Xylanases cleave the β-1,4 glycosidic linkages between xylose residues in the backbones of xylans, providing one of the most important enzymatic activities required for depolymerization of the hemicellulosic constituent of plant cell walls. Their action, together with other accessory enzymes, has been reported to improve cellulose conversion by removing hemicellulose and increasing the accessibility of the substrate to cellulases. Goldbeck et al. (2016) investigated the effect of six hemicellulases on pretreated sugarcane bagasse and showed that the endo-1,4-xylanases and the feruloyl esterase effectively broke down hemicelluloses and increased saccharification of pretreated lignocellulose by 24 %.

The *Scytalidium* strain was able to produce 0.73 IU/mg of pectinase and 0.71 IU/mg of linear arabinan. Delabona et al. (2013) demonstrated that the supplementation of *Trichoderma harzianum* enzymatic extract with pectinase and α-L-arabinofuranosidase increases the hydrolysis efficiency up to 116 %. The enzymatic activity values reported here are similar or superior to those reported for *Penicillium echinulatum (*Delabona et al. 2013). The biomass saccharification by *P. echinulatum* cocktail was superior when compared to the commercial preparation such as cellulase from *Trichoderma viride* (C9422, Sigma-Aldrich, USA) and Cellic Ctec 2 (Novozymes, Denmark) because of the presence of these two important enzymatic activities: pectinase and linear arabinanase (Delabona et al. 2013). This evidence reveals the real potential of biomass saccharification attributed to the novel strain reported in this work.

Biomass saccharification

Figure 5 portrays the temporal profiles of glucose, cellobiose, and xylose release due to Celluclast (A) and the new strain *Scytalidium* MIBA 247 (B) catalysis. The amounts of glucose released were superior for *Scytalidium* crude enzymatic extract in comparison with Celluclast. In 72 h of alkaline pretreated bagasse hydrolysis, the *Scytalidium* sp extract was able to release 43.57±0.58 g/L of glucose (containing 9.4 $IU/g_{bagasse}$ of β-glucosidase), while the commercial enzyme preparation released 33.52±0.81 g/L of glucose (containing 10.4 $IU/g_{bagasse}$ of β-glucosidase). It is interesting to note that the novel *Scytalidium* crude enzymatic extract in-house produced presented a positive effect on the hydrolysis of pretreated sugarcane bagasse, probably because of the action of accessory enzymes, as shown in Table 3. These enzymes probably contribute to the opening of the cellulose fibers and improve the access of cellulolytic
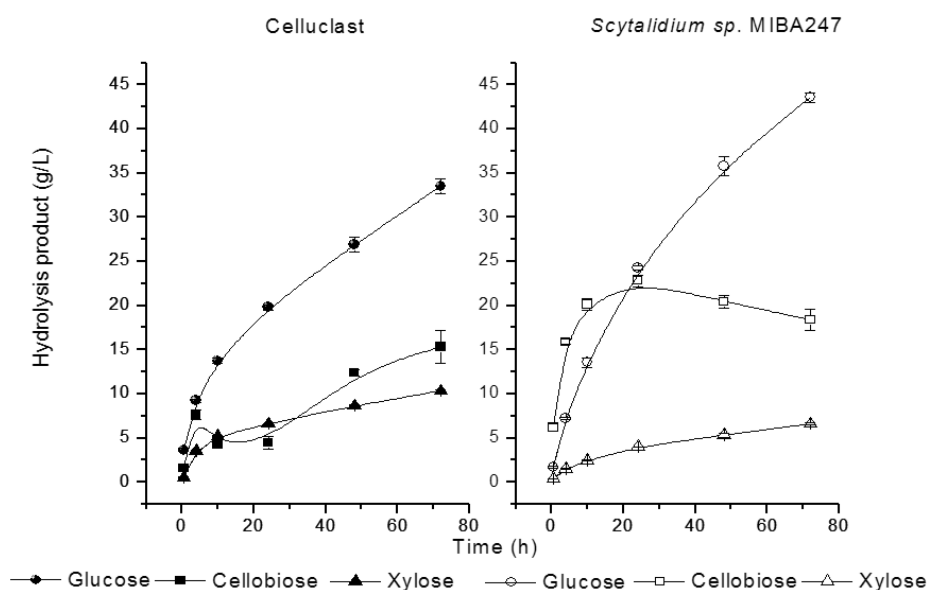

Figure 5. Profiles of glucose, cellobiose and xylose release over time during hydrolysis experiments in shake flasks performed using Celluclast (A) and *Scytalidium* sp. (B).

enzymes. As mentioned before, the correct set of accessory enzymes required for biomass degradation is also highly dependent on the choice of pretreatment technology as the chemical structures and linkages of the plant cell wall components are often modified after the pretreatment step (Alvira et al., 2011).

The low concentrations of xylose released by this new strain were due to the low hemicellulose degrading enzyme activities in its extract when compared to the commercial enzyme preparation, since the alkaline pretreatment bagasse has significant hemicellulose content (21.1 %, on a dry basis). The yield in hemicellulose after 72 h was 0.25g of hemicellulose equivalent from xylose/g hemicellulose and 0.43g of hemicellulose equivalent from xylose/g hemicellulose, respectively, for MIBA (containing 125.8 IU/$g_{bagasse}$ of xylanase) and Celluclast (containing 49.9 IU/$g_{bagasse}$ of xylanase). This conversion value was 74 % lower when compared to the commercial enzyme preparation.

There was a higher accumulation of cellobiose when *Scytalidium* MIBA 247 cocktail was applied to the conversion of biomass (Fig. 5B) in comparison to Celluclast (Fig. 5A). After 72 h, the cellobiose concentration obtained (with 125.77 UI of β-glucosidase/$g_{bagasse}$) was 18.39 ± 1.2 g/L, while for the commercial enzyme preparation the cellobiose concentration was 15.35±1.86 g/L. The yield in cellulose achieved after 72 h was 0.81g of cellulose equivalent from glucose and cellobiose/g hemicellulose and 0.71 g cellulose equivalent from glucose and cellobiose/g hemicellulose, respectively, for MIBA and Celluclast.

The addition of β-glucosidases to the enzymatic cocktail is absolutely necessary not only because glucose is the desirable final product, but also because of the aim to reduce the cellobiose inhibition of endo-β-1,4-glucanases (EG) and cellobiohydrolases (CBH). Therefore, a low level of β-glucosidases activity limits cellulose hydrolysis, resulting in a low level of saccharification.

The commercial β-glucosidases (Novozyme 188) are indeed a suitable supplementary enzyme for reinforcing *Trichoderma reesei* enzymatic cocktail (widely used in industrial applications), which remains the traditionally favorable enzyme mixture for lignocellulosic materials degradation (Treebupachatsakul et al., 2016). Addition of β-glucosidase from *Scytalidium lignicola* to a saccharification system resulted in an increase of 30 % in reducing sugar (Desai and Ray, 1986). Therefore, the addition of exogenous β-glucosidase to the *Scytalidium* crude enzymatic extract could improve not only the efficiency of cellulose hydrolysis, but also the rate of glucose production.

The enzymatic cocktail produced by *Scytalidium* is capable of hydrolyzing 62 % of the alkaline pretreated

bagasse, being superior to the hydrolysis performed with the commercial enzyme preparation (48 %). Although the enzymatic activity per volume of the commercial cocktail is higher than the home-made one, the load of enzymes added to the hydrolysis experiments were the same (10 FPU per gram of bagasse). In this case, it is possible to evaluate the efficiency of each cocktail and compare the influence of their composition on the conversion, regardless of the concentration in the stock solution. The new enzymes identified here will be explored in terms of substrate specificities and their potential to hydrolyze substrates in order to develop a feasible enzymatic cocktail with superior saccharification yields.

CONCLUSIONS

This new strain of *Scytalidium* isolated from the Amazon rainforest appeared to be a potential candidate for enzyme production using delignified hydrothermal sugarcane bagasse and yeast extract as substrate. Both of these materials are in ready supply in bioethanol production plants, allowing the reduction of the costs for enzyme production. The better performance of the *Scytalidium* crude enzymatic extract compared to commercial enzymes for sugarcane bagasse hydrolysis suggests that the novel strain secreted a satisfactory set of the accessory enzymes required for biomass degradation.

ACKNOWLEDGEMENTS

NOMENCLATURE

ANOVA	Analysis of Variance
AB	Alkaline-pretreated Bagasse
CBH	Cellobiohydrolase
CCR	Carbon Catabolite Repression
CCRD	Central Compound Rotational Design
CE	Commercial Cellulose Celufloc 200™
CMC	Carboxymethylcellulose
DHB	Delignified-Hydrothermal Pretreated Bagasse
DNS	Dinitrosalicylic Acid
DSB	Delignified Hydrothermal Pretreated Bagasse

EG	Endo-β-1,4-glucanases
FPAse	Filter Paper Activity
FPU	Filter Paper Unit
GH	Glycosyl Hydrolase
HB	Hydrothermal Pretreated Bagasse
IR	Infrared
ITS	Internal Transcribed Spacer
IU	International Unit
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
p-NP	ρ-Nitrophenol
SB	Steam-pretreated Bagasse
SM	Supplementary Material
SUC	Sucrose
YE	Yeast Extract

REFERENCES

Alvira, P., Negro, M.J., Ballesteros, M., Effect of endoxylanase and α-L-arabinofuranosidase supplementation on the enzymatic hydrolysis of steam exploded wheat straw. Bioresour. Technol. 102, 4552-4558 (2011). https://doi.org/10.1016/j.biortech.2010.12.112

Balaa, B. Al, Brijs, K., Gebruers, K., Vandenhaute, J., Wouters, J., Housen, I., Xylanase XYL1p from Scytalidium acidophilum: Site-directed mutagenesis and acidophilic adaptation. Bioresour. Technol. 100, 6465-6471 (2009). https://doi.org/10.1016/j.biortech.2009.06.111

Barua, P., Barua, S., Borkakoty, B., Mahanta, J., Onychomycosis by Scytalidium dimidiatum in green tea leaf pluckers: Report of two cases. Mycopathologia 164, 193-195 (2007). https://doi.org/10.1007/s11046-007-9024-9

Bradford, M.M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254 (1976). https://doi.org/10.1016/0003-2697(76)90527-3

Braga, C.M.P., Delabona, P.D.S., Lima, D.J.D.S., Paixão, D.A.A., Pradella, J.G.D.C., Farinas, C.S., Addition of feruloyl esterase and xylanase produced on-site improves sugarcane bagasse hydrolysis. Bioresour. Technol. 170, 316-324 (2014). https://doi.org/10.1016/j.biortech.2014.07.115

Bussamra, B.C., Freitas, S., Costa, A.C. da, Improvement on sugar cane bagasse hydrolysis using enzymatic mixture designed cocktail. Bioresour. Technol., 187, 173-181 (2015). https://doi.org/10.1016/j.biortech.2015.03.117

Carlos, J., Silva, R., Henrique, L., Guimarães, S., Carlos, J., Salgado, S., Prazeres, R., Furriel, M., Polizeli, M.L.T.M., Rosa, J.C., Jorge, J.A., Purification and biochemical characterization of glucose - cellobiose-tolerant cellulases from S cytalidium thermophilum. Folia Microbiol 58, 561-568 (2013). https://doi.org/10.1007/s12223-013-0245-7

de Souza, A.P., Leite, D.C.C., Pattathil, S., Hahn, M.G., Buckeridge, M.S., Composition and Structure of Sugarcane Cell Wall Polysaccharides: Implications for Second-Generation Bioethanol Production. Bioenergy Res. 6, 564-579 (2013). https://doi.org/10.1007/s12155-012-9268-1

Delabona, P.S., Pirota, R.D.P.B., Codima, C.A., Tremacoldi, C.R., Rodrigues, A., Farinas, C.S., Using Amazon forest fungi and agricultural residues as a strategy to produce cellulolytic enzymes. Biomass and Bioenergy 37, 243-250 (2012). https://doi.org/10.1016/j.biombioe.2011.12.006

Delabona, P.S., Lima, D.J., Robl, D., Rabelo, S.C., Farinas, C.S., da Cruz Pradella, J.G., Enhanced cellulase production by Trichoderma harzianum by cultivation on glycerol followed by induction on cellulosic substrates. J. Ind. Microbiol. Biotechnol. 43, 617-626 (2016). https://doi.org/10.1007/s10295-016-1744-8

Delabona, P.S., Cota, J., Hoffmam, Z.B., Paixão, D.A.A., Farinas, C.S., Cairo, J.P.L.F., Lima, D.J., Squina, F.M., Ruller, R., Pradella, J.G. da C., Understanding the cellulolytic system of Trichoderma harzianum P49P11 and enhancing saccharification of pretreated sugarcane bagasse by supplementation with pectinase and α-l-arabinofuranosidase. Bioresour. Technol. 131, 500-507 (2013). https://doi.org/10.1016/j.biortech.2012.12.105

Delabona, P.S., Farinas, C.S., da Silva, M.R., Azzoni, S.F., Pradella, J.G.D.C., Use of a new Trichoderma harzianum strain isolated from the Amazon rainforest with pretreated sugar cane bagasse for on-site cellulase production. Bioresour. Technol. 107, 517-521 (2012). https://doi.org/10.1016/j.biortech.2011.12.048

Desai, J.D., Ray, R., Immobilization of B-Glucosidase from Scytalidium lignicola on Chitosan. J. Ferment. Technol., 64, 255-258 (1986). https://doi.org/10.1016/0385-6380(86)90108-1

Dos Reis, L., Ritter, C.E.T., Fontana, R.C., Camassola, M., Dillon, A.J.P., Statistical optimization of mineral salt and urea concentration for cellulase and xylanase production by Penicillium echinulatum in submerged fermentation. Braz. J. Chem. Eng., 32, 13-22 (2015). https://doi.org/10.1590/0104-6632.20150321s00003099

Dos Santos Costa, P., Büchli, F., Robl, D., da Silva Delabona, P., Rabelo, S.C., da Cruz Pradella, J.G., Enhancement of Penicillium echinulatum glycoside hydrolase enzyme complex. J. Ind. Microbiol. Biotechnol. 43, 1-13 (2016). https://doi.org/10.1007/s10295-016-1746-6

Efron, B., Tibshirani, R.J., An Introduction to the Bootstrap. CRC Press (1994). https://doi.org/10.1111/1467-9639.00050

Florencio, C., Couri, S., Farinas, C.S., Correlation between Agar Plate Screening and Solid-State Fermentation for the Prediction of Cellulase Production by Trichoderma Strains, Enzyme Research, 2012, 1-7 (2012). https://doi.org/10.1155/2012/793708

Ghose, T.K., Measurement of cellulase activities. Pure Appl. Chem. 59, 257-268 (1987). https://doi.org/10.1351/pac198759020257

Goldbeck, R., Damásio, A.R.L., Gonçalves, T. a., Machado, C.B., Paixão, D. a. a., Wolf, L.D., Mandelli, F., Rocha, G.J.M., Ruller, R., Squina, F.M., Development of hemicellulolytic enzyme mixtures for plant biomass deconstruction on target biotechnological applications. Appl. Microbiol. Biotechnol., 98, 8513-8525 (2014). https://doi.org/10.1007/s00253-014-5946-6

Goldbeck, R., Gonc, T.A., Damásio, A.R.L., Brenelli, L.B., Wolf, L.D., Paixão, D.A.A., Rocha, G.J.M., Squina, F.M., Journal of Molecular Catalysis B : Enzymatic Effect of hemicellulolytic enzymes to improve sugarcane bagasse saccharification and xylooligosaccharides production 131, 36-46 (2016). https://doi.org/10.1016/j.molcatb.2016.05.013

Hendy, N., Wilke, C., Blanch, H.,. Enhanced cellulase production using solka floc in a fed-batch fermentation. Biotechnol. Lett. 4, 785-788 (1982). https://doi.org/10.1007/BF00131153

Ilmen, M., Saloheimo, A., Onnela, M.-L., Penttila, M.E., Regulation of cellulase gene expression in the filamentous fungus *Trichoderma reesei*. Appl. Environ. Microbiol. 63, 1298-1306 (1997).

Johnson, E., Integrated enzyme production lowers the cost of cellulosic ethanol. Biofuels, Bioprod. Biorefining 6, 246-256 (2016).. doi:10.1002/bbb

Kaur, J., Chadha, B.S., Saini, H.S., Regulation of cellulase production in two thermophilic fungi *Melanocarpus sp* . MTCC 3922 and *Scytalidium thermophilum* MTCC 4520, Enzyme and Microbial Technology, 38, 931-936 (2006). https://doi.org/10.1016/j.enzmictec.2005.08.036

Kimura, M., A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111-120 (1980). https://doi.org/10.1007/BF01731581

Kirk, P.M., Cannon, P.F., Minter, D.W., Stalpers, J.A., Dictionary of The Fungi. Mycol. Res. 106, 507-508 (2008). https://doi.org/10.1017/S0953756202216160

Kiss, L., Limits of nuclear ribosomal DNA internal transcribed spacer (ITS) sequences as species barcodes for Fungi. Proc. Natl. Acad. Sci. 109, E1811-E1811 (2012). https://doi.org/10.1073/pnas.1207143109

Kubicek, C.., Fungi and Lignocellulosic Biomass. Wiley Blackwell 304 (2012).

Maeda, R.N., Serpa, V.I., Rocha, V.A.L., Mesquita, R.A.A., Anna, L.M.M.S., De Castro, A.M., Driemeier, C.E., Pereira, N., Polikarpov, I., Enzymatic hydrolysis of pretreated sugar cane bagasse using Penicillium funiculosum and Trichoderma harzianum cellulases. Process Biochem. 46, 1196-1201 (2011). https://doi.org/10.1016/j.procbio.2011.01.022

Miller, G.L., Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Anal. Chem. 31, 426-428 (1959). https://doi.org/10.1021/ac60147a030

Moreno, M.L., Piubeli, F., Bonfá, M.R.L., García, M.T., Durrant, L.R., Mellado, E., Analysis and characterization of cultivable extremophilic hydrolytic bacterial community in heavy-metal-contaminated soils from the Atacama Desert and their biotechnological potentials. J. Appl. Microbiol. 113, 550-559 (2012). https://doi.org/10.1111/j.1365-2672.2012.05366.x

Nakari-Setala, T., Penttila, M., Production of Trichoderma cellulase on glucose containing media. Appl. Environ. Microbiol 61, 3650-3655 (1995).

Nascimento, V.M., Nakanishi, S.C., Rocha, G.J.M., Rabelo, S.C., Pimenta, M.T.B., Rossel, C.E. V., Effect of Anthraquinone on Alkaline Pretreatment and Enzymatic Kinetics of Sugarcane Bagasse Saccharification: Laboratory and Pilot Scale Approach. ACS Sustainable Chemistry & Engineering, 4, 3609-3617 (2016). https://doi.org/10.1021/acssuschemeng.5b01433

Nilsson, R., Kristiansson, E., Ryberg, M., Hallenberg, N., Larsson, K.-H., Intraspecific ITS variability in the kingdom Fungi as expressed in the international sequence databases and its implications for molecular species identification. Evol. Bioinforma. 4, 193-201 (2008). https://doi.org/10.4137/EBO.S653

Padmavathi, T., Agarwal, P., Nandy, V., Exploring marine fungal strains for cellulase production 3, 3602-3613 (2012).

Robl, D., dos Santos Costa, P., Büchli, F., da Silva Lima, D.J., da Silva Delabona, P., Squina, F.M., Pimentel, I.C., Padilla, G., da Cruz Pradella, J.G., Enhancing of sugar cane bagasse hydrolysis by Annulohypoxylon stygium glycohydrolases. Bioresour. Technol. 177, 247-254 (2015). https://doi.org/10.1016/j.biortech.2014.11.082

Ruegger, M.J.S., Tauk-tornisielo, S.M., Atividade da celulase de fungos isolados do solo da Estação Ecológica de Juréia-Itatins, São Paulo, Brasil. Revista Brasil. Bot., 2, 205-211 (2004).

Saitou, N., Nei, M., The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molec. Biol. Evol. 4, 406-425 (1987). https://doi.org/10.1093/oxfordjournals.molbev.a040454

Suto, M., Tomita, F., Induction and catabolite repression mechanisms of cellulase in fungi. J. Biosci. Bioeng. 92, 305-311 (2001). https://doi.org/10.1016/S1389-1723(01)80231-0

Tamura, K., Dudley, J., Nei, M., Kumar, S., MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596-1599 (2007). https://doi.org/10.1093/molbev/msm092

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., The CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876-4882 (1997). https://doi.org/10.1093/nar/25.24.4876

Treebupachatsakul, T., Nakazawa, H., Shinbo, H., Fujikawa, H., Nagaiwa, A., Ochiai, N., Kawaguchi, T., Nikaido, M., Totani, K., Shioya, K., Shida, Y., Morikawa, Y., Ogasawara, W., Okada, H., Heterologously expressed Aspergillus aculeatus b -glucosidase in Saccharomyces cerevisiae is a cost-effective alternative to commercial supplementation of b - glucosidase in industrial ethanol production using Trichoderma reesei cellulases. J. Biosci. Bioeng. 121, 27-35 (2016). https://doi.org/10.1016/j.jbiosc.2015.05.002

White, T.J., Bruns, S., Lee, S.J.W.T., Taylor, J.L., Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications, 18, 315-322 (1990). doi:citeulike-article-id:671166.

Zhang, Y.-H.P., Schell, D.J., McMillan, J.D., Methodological analysis for determination of enzymatic digestibility of cellulosic materials. Biotechnol. Bioeng. 96, 188-194 (2007). https://doi.org/10.1002/bit.21178