Co-Culture of *Trichoderma Reesei, Talaromyces* Sp. and *Aspergillus* Spp. Produces A Multi-Enzyme Cocktail for the Hydrolysis of Sugarcane Bagasse Pretreated with Piperonilic Acid (PIP) and Methylenedioxycinnamic Acid (MDCA)

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Abstract

PURPOSE: The global energy matrix is primarily based on fossil fuels and alternatives for the production of renewable energy are necessary. The second-generation ethanol (2G ethanol) is such alternative. 2G ethanol is produced through the fermentation of sugar released from the enzymatic hydrolysis of lignocellulosic biomass. However, this process is still costly. Improvements should include the use of less expensive biomass pre-treatments and enzymatic cocktails produced by the co-cultivation of filamentous fungi.

METHODS: For the production of synergistic holocellulolytic enzymes, Aspergillus brasiliensis , A. fumigatus var. niveus , Trichoderma reesei and Talaromyces sp. were co-cultivated on sugarcane bagasse modified in the lignin synthesis pathway. This bagasse was pretreated with piperonilic acid (PIP) and methylenedioxycinnamic acid (MDCA).

RESULTS: The enzymatic cocktail produced by the co-culture showed the highest hydrolysis efficiency. The best hydrolysis condition was at 50°C and pH 4.0. Talaromyces sp. and T. reesei demonstrated antagonism only between them.

CONCLUSION: Enzymatic cocktails produced through the co-cultivation of filamentous fungi are a concrete step towards increasing yields for the 2G ethanol industry.

Statement Of Novelty

The global economy is dependent on unsustainable fossil fuels. Climate change is an indisputable threat to humanity and societies must transition to sustainable energy sources. The second-generation (2G) ethanol is an alternative, but high production costs still impede the scaling out of this technology. Biomass pretreatment and the production of lignocellulolytic enzymes are the most costly steps. This study approaches both issues. The co-cultivation of four fungi produced a multi-enzyme cocktail capable of degrading lignocellulosic biomass (sugarcane bagasse). The pretreatment utilized has been co-opted from studies on the effects of allelochemicals in plant development. Lignin synthesis inhibitors have shown to disrupt lignin deposition in the plant cell wall and might represent a new biochemical pretreatment for lignocellulosic materials.

1. Introduction

The planetary boundaries for a safe and sustainable human existence were first established in 2009 [1]. They were revisited and updated in 2015 [2] ahead of the COP21 that culminated in the Paris Agreement [3]. Because of the Covid-19 pandemic, the global primary energy consumption declined 4.5% and carbon emissions tanked 6.3% in 2020 [4]. However, more than 80% of the world's energy matrix still derives from fossil fuels [5]. The negotiations from COP26 have fallen short [6, 7] and the 1.5ºC Pathway is no longer attainable by 2050 [8]. Indeed, the demands for oil and gas are expected to peak in the coming decades [8]. The consumption of fossil fuels is the largest source of greenhouse gases (GHGs) emissions [9], and climate change is no longer a grim possibility, but an alarming reality [10]. The effects of a warmer climate are wide ranging, as they accelerate the loss of biodiversity, desertification, sea level rising, to list a few [11, 12]. Renewable energy sources are then necessary [13] and the second generation (2G) ethanol is one alternative [14].

2G ethanol is produced through the enzymatic hydrolysis of lignocellulosic biomass [15]. This biomass (i.e. the plant cell wall) is constituted primarily of cellulose, hemicellulose, and lignin [16]. Through pretreatments and enzymatic reactions, the plant cell wall can be broken down into fermentable sugars (i.e. saccharification), that in turn are converted to ethanol (i.e. fermentation) [17]. But, lignocellulosic materials are recalcitrant and present variable composition among different plant species [18, 19]. Tailored pretreatments and heterogeneous enzymatic cocktails are then needed for efficient biomass degradation [20, 21].

Traditional biomass pretreatments often employ harsh and costly physicochemical conditions, as they use concentrated acid or alkaline solutions and other solvents [22]. Lignin synthesis inhibitors, such as piperonilic acid (PIP) and methylenedioxycinnamic acid (MDCA), represent a viable and eco-friendly pretreatment alternative. These compounds inhibit the phenylpropanoid pathway, ultimately reducing the amount of lignin in the plant cell wall [23]. PIP irreversibly blocks cinnamate 4-hydroxylase and MDCA competitively inhibits 4-coumarate:CoA ligase [24, 25]. As the lignin synthesis is disrupted, the use of such inhibitors is expected to increase biomass digestibility by loosening and exposing cellulose fibers.

The saccharification process comes after an effective pretreatment. Lignocellulolytic enzymes derived from fungi are extensively used in this step [26]. Axenic fungal cultures produce crude extracts [27] or heterologous proteins [28] that are capable of degrading different biomass sources. Several fungi can be grown separately and have their extracts subsequently combined into multi-enzyme cocktails as well [29]. A cost-effective alternative is the co-culture of fungi [30], an approach that is also called co-cultivation or microbial/fungal consortium [31, 32]. These consortia grow synergistically and produce high-performance enzymatic cocktails [33] along with several secondary metabolites of value to other industries [34].

Aspergillus and *Trichoderma* species are model organisms in the production of lignocellulolytic enzymes [35]. They secrete cellulases and hemicellulases that can be produced in large-scale [36, 37], but oftentimes do not produce key accessory enzymes in sufficient amounts [38]. Here, we aimed to produce an enzymatic cocktail via the co-cultivation of *Trichoderma reesei,Aspergillus brasiliensis*, *Aspergillus fumigatus var. niveus*, and *Talaromyces* sp. The multi-enzyme cocktail was applied in the saccharification of sugarcane bagasse pretreated with piperonilic acid (PIP) and methylenodyoxicinnamic acid (MDCA).

2. Materials And Methods

2.1 Fungal strains

The strains are deposited at the fungal collection of the Microbiology and Cell Biology laboratory at FFCL/USP-RP, Ribeirão Preto, Brazil. We used the model species *T. reesei* RUT C30 (ATCC 56765), *A. brasiliensis* and *A. fumigatus* var. *niveus* [39], and a putative new species of *Talaromyces* sect. *Talaromyces.*

2.2 Confrontation assay

A confrontation assay was conducted to analyze the growth and behavior of the fungal strains when coexisting in the same environment. The assays used Petri dishes with PDA culture media cut in a cross format. Each fungal species was inoculated in each tip of the cross and in the center of it, leaving one of the tips without any fungi serving as an experimental control.

2.3 Optimization of enzyme extract production

The fungal strains were cultivated in 50 mL of Minimum Media (composed of trace elements and nitrate salts, pH = 6.5) [35], with 1% of biomass (0.5 g of *in natura* sugarcane bagasse), in duplicate. A suspension of spores (10^7) was inoculated, and the flasks were incubated at 30 °C, 120 rpm, for 5 days. Then, 10 mL of the extract was centrifuged at 4.000 rpm and the supernatant was collected and stored at 4 °C.

2.4 Total protein determination

Total protein determination followed the modified method of Bradford [40]. The procedure was performed in a 96-well plate, in triplicates. Each reaction had a total of 200 µL, with 40 µL of the Coomassie Brilliant Blue BG-250 (BioRad®) reagent and 160 µL of the extract. The blank was made with 160 µL of water and 40 µL of the reagent only. The quantification was estimated by absorbance at 595 nm. The standard curve was calculated using bovine serum albumin (Sigma). The protein unit was defined as µg of protein/mL.

2.5 SDS-PAGE

Protein electrophoresis of the extracts was performed by the SDS-Page method. The 12% run gel and the 5% stacking gel were prepared in sufficient volume for a 1 mm plate. The enzyme extracts were concentrated in SpeedVac. 10 µL of the extract and 10 µL of the loading dye with 2-mercaptoethanol were mixed and boiled at 100 °C, applied in the gel, and run at 120 V. After the run, the gel was colored with Coomassie Blue dye for later visualization of the bands.

2.6 Enzymatic assays

The enzymatic assays were performed in 96-well plates, with each reaction containing 10 µL of 50 mM sodium acetate buffer (pH = 5.0), 15 µL of enzyme extract, 25 µL of substrate, and subsequent addition of 50 µL of 3,5-dinitrosalicylic acid (DNS), or Na_2CO_3, totaling 100 µL. Natural substrates were used for cellulases (1% CMC and 1% Avicel), for xylanases (Xylan from Beechwood 1%) and for xyloglucanases (Xyloglucan 0.5%). Synthetic substrates were used for endo and exocellulases (2mM pnp-β-D-cellobioside) and for β-glucosidases (2mM pnp-β-D-glucopyranoside). The method from Miller [41] was used for the determination of reducing sugars released by the degradation of natural substrates. For the synthetic substrates, the reactive agent used was 0.2 M Na_2CO_3. The reactions were incubated at 50 °C for 30 min, and then the revealing reagents were added. In the DNS assay, after its addition to the reactions, they were submitted for another heating at 98 °C for 5 min. There was a blank reaction for each enzymatic reaction. The plates were read at 540 nm and 410 nm for the natural and synthetic substrates, respectively.

The standard curve was made using cellobiose, xylose or glucose to calculate the enzymatic activity on natural substrates. Paranitrophenol (pnp) was used in the standard curve for the synthetic substrates. The unit of enzyme activity was defined as the amount of enzyme able to release 1 µmol of product per minute under the assay conditions.

2.7 Biomass hydrolysis by the enzymatic cocktail produced from fungi grown in sugarcane bagasse with modified lignin synthesis pathway

2.7.1 Production of extracts from each sample and its combinations

Separate enzyme extracts were prepared for each fungus as described above, in item 2.4. The fungi were cultivated in three distinct types of sugarcane bagasse. Two types were pretreated and the third type was *in natura* (non-treated) bagasse used for the experimental control. The pretreated bagasse was obtained with the use of lignification inhibitors, piperonyl acid (PIP) and methylenedioxycinnamic acid (MDCA).

The enzymatic extracts had their total proteins quantified and further analyzed on polyacrylamide gel as described above (item 2.6).

This initial hydrolysis was performed in triplicates, in 1 mL 96-well plates containing 500 µL of 50 mM sodium acetate buffer (pH = 5.0) and 500 µL of enzyme extract and 3% sugarcane bagasse. The extracts of different fungi were combined as follow: *A. brasiliensis* + *T. reesei*, *A. fumigatus var. niveus* + *T. reesei*, *Talaromyces* sp. + *T. reesei*, *A. brasiliensis* + *A. fumigatus var. niveus* + *Talaromyces* sp.; *A. brasiliensis* + *A. fumigatus var. niveus* + *T. reesei* + *Talaromyces* sp. The volumes of each extract in the reaction were proportional to the number of fungi mixed in, in order to totalize 500 µL of extract

(e.g. 250 µL of *A. fumigatus var. niveus* extract + 250 µL of *T. reesei* extract). For each reaction, a blank was determined, using water instead of the enzyme extract.

The plates were incubated under agitation at 50 °C, for 24 h. At the end, the plates were centrifuged at 4,000 rpm, for 10 minutes. Afterward, the released reducing sugars were measured by the DNS method as described above (item 2.7).

2.7.2 Co-culture

The co-cultures followed the steps mentioned in items 2.3 and 2.7.1 and used the same three different types of bagasse mentioned above. For the co-cultures, the fungi were inoculated together using an equal volume of the spore solution of each fungus, for a total of 1 mL of inoculum. In duplicate, there were five combinations for each type of bagasse: *A. brasiliensis* + *T. reesei*, *A. fumigatus var. niveus* + *T. reesei*, *Talaromyces* sp. + *T. reesei*, *A. brasiliensis* + *A. fumigatus var. niveus* + *Talaromyces* sp.; *A. brasiliensis* + *A. fumigatus var. niveus* + *T. reesei* + *Talaromyces* sp.

The samples also had their total proteins quantified and analyzed on SDS-PAGE gel. The hydrolysis and enzymatic assays were performed as described above.

2.8 Optimization of hydrolysis assays for the produced cocktail

The optimal pH and temperature of the best performing cocktail were determined by a similar plate assay as described in 2.8. For the optimal pH, the hydrolysis assays were conducted in a pH range of 3–8, using a McIlvaine buffer. For the optimal temperature, the assays were conducted in a range of 30–80 °C, with an interval of 10 °C. The released reducing sugars were determined by the DNS method from Miller, as described above.

3. Results And Discussion

3.1. Standardization of the enzymatic production

3.1.1. Protein profile

A. brasiliensis, *A. fumigatus var. niveus*, and *Talaromyces* sp., had a similar protein amount for all extracts when the same volume was used in the reaction. The exception was *T. reesei*, which secreted slightly higher amounts of protein (Fig. 1a). The same was observed in the co-cultivation, indicating that the sugarcane bagasse induced a similar amount of proteins in all situations (Fig. 1b).

All these data were validated by SDS-PAGE (data not shown). However, it was observed, by SDS-PAGE, a different protein profile among different extracts, which allows us to infer that the substrate (sugarcane bagasse) differentially induces the production of proteins across them.

3.2.2. Enzymatic activity profile

The screening of enzymatic activity counts as an important analysis to achieve a catalytic profile from the microorganisms [42]. The substrates chosen in this investigation, xylan from beechwood, avicel, carboximetilcellulose (CMC), xyloglucan, pnp-β-D-glucopyranoside and pnp-β-D-cellobioside, represented a good diversity to screen the main catalysts that attack the sugarcane bagasse cell wall [43].

A. brasiliensis and *A. fumigatus v. niveus* had hemicellulolytic, but low cellulolytic activity (Table 1). Although *A. brasiliensis* is very similar to *A. niger*, its glucosidase activity is very low, demonstrating the need to add some other good producer in the enzymatic blend. *T. reesei* RutC30 is known to secret cellulolytic enzymes [44, 45]. *Talaromyces* sp. presented a considerable activity of cellulases and hemicellulases as well (Table 1).

Fungi from the section Nigri are known to produce xylanases and β-glucosidases [46, 47]. So, *A. brasiliensis* was added to the cocktail to supplement the cellulolytic cocktail secreted by *T. reesei*. *A. fumigatus var. niveus* is a good hemicellulase producer as well [48]. *Talaromyces* sp. was added because several species of this genus secrete hemicellulases and cellulases (including β-glucosidades) [49, 50, 51].

3.3. Confrontation assays for co-culture validation

Prior to the co-culture, the fungi were evaluated for their ability to grow together. *A. brasiliensis* presented high sporulation and very fast growth like other black fungi such as *A. niger*, *A. saccharolyticus* and *A. carbonarius* [52]. Plates inoculated with *A. brasiliensis* were totally covered, including the tip used as control. The colonies of *A. fumigatus var. niveus* and *T. reesei* were not inhibited (Online Resource 1). Theconfrontation assayof *Talaromyces* sp.was performed without inoculating *A. brasiliensis* because of its very fast growth. All fungi grew well and *A. fumigatus var. niveus* grew normally in the presence of *Talaromyces sp.* However, *Talaromyces* sp. exhibited a small inhibition halo when in contact with *T. reesei*, indicating a possible competition between them (Online Resource 1).

3.4. Hydrolysis of the pretreated (lignin-inhibited) sugarcane bagasse

Under co-cultivation, the cocktails produced on PIP-pretreated bagasse promoted hydrolysis under all conditions (Fig. 2a). The cocktail produced by *Talaromyces* sp. + *T. reesei* exhibited the highest release of reducing sugars when applied to the MDCA-pretreated bagasse (Fig. 2b). The MDCA pretreatment probably made the cellulose skeleton more accessible to cellulolytic enzymes.

The cocktail produced by *Talaromyces* sp. with *T. reesei*, on the control and MDCA-pretreated bagasse, exhibited lower levels of hydrolysis when applied onto treated and untreated bagasse. Co-cultures of *T. reesei* with *Aspergillus* species have presented low hydrolysis efficiency before [52]. This could be explained by the possible competition between these two fungi (as observed in the confrontation assay), or by the production of holocellulolytic enzymes containing carbohydrate-binding modules (CBMs). Although CBMs are important to hydrolytic activity [53], they can also decrease enzymatic hydrolysis by causing the enzymes to adhere to lignin [54]. The control (*in natura* bagasse) and the MDCA bagasse could represent a lignin-rich surface and a substrate where lignin is more readily available, respectively.

The co-cultivation of all fungi grown in both PIP- and MDCA-pretreated bagasse showed good hydrolytic capacity (Fig. 2c). Microbial co-cultivation can be a cost effective alternative for industrial processes [55]. Co-culturing fungi decreases enzyme production costs, as inputs (reagents, electricity and humanpower) are better used [56]. Microbial consortia also minimizes the incubation/fermentation time while maintaining high levels of enzyme production [57].

Combining enzymatic extracts produced by the fungi after being cultivated separately exhibited similar results and validated the efficiency of the co-cultivation strategy. The extracts obtained from fungi grown in PIP-pretreated bagasse had the best results (approximately 1.1 µmol/mL). The combination of all extracts released 2 µmol/mL of reducing sugars from the MDCA-pretreated bagasse (Fig. 3a). The combination of *Talaromyces* sp. and *T. reesei* extracts had a higher hydrolysis efficiency in the extracts produced using the PIP-pretreated bagasse. The extracts produced using the control and MDCA-pretreated bagasse yielded, respectively, 0.9 µmol mL and 1.3 µmol/mL of released reducing sugars from the MDCA-pretreated bagasse. These results are quite higher than those from the co-cultivation, which corroborates the possible competition between these two strains when grown together. As the fungi grew separately under ideal conditions (i.e. without interspecific competition), they might have produced extracts with higher amounts of holocellulolytic enzymes that could yield a more efficient hydrolysis when combined (Fig. 2b). Although the combination of the separate extracts exhibited similar results to the co-cultivation, the latter strategy is still more advantageous as it is more cost-effective.

The sugarcane pretreatments with lignin synthesis inhibitors alter the structure and amount of lignin, but the concentration of cellulose, hemicellulose and other components remains intact. Some varieties of sugarcane also have a modified concentration of other plant cell wall components. Applying the PIP- and MDCA-pretreatments on cellulose-rich sugarcane varieties, such as the energy cane [58], would be an interesting strategy for the bioethanol industry. Combining these technologies with the use holocellulolytic cocktails produced through the co-cultivation of fungi can only augment the current 2G bioethanol industrial processes.

3.5. Optimization of hydrolysis of the co-cultured PIP-extract

The co-cultivation of *A. brasiliensis*, *A. fumigatus var. niveus*, *T. reesei* and *Talaromyces* sp.in submerged fermentation using PIP-pretreated sugarcane bagasse as the only carbon source, presented the best hydrolysis efficiency in all bagasse (Fig. 3a). At 50 °C, the optimal pH was 4.0, showing a prominent increase in the hydrolysis efficiency of all bagasse tested in this study (Fig. 4a and Fig. 4b).

4. Conclusions

The co-cultivation of fungal species in the pretreated bagasse was confirmed as a good alternative for the enzymatic production, leading to obtaining a cocktail capable of hydrolyzing different types of biomass efficiently. Thus, this strategy together with biomass pretreatments (PIP and MDCA), that favor the accessibility of enzymes to the cellulose skeleton, can increase the efficiency of this bioprocess. However, the efficiency of extract combinations in an enzyme blend is not discarded, and it is a good alternative for the hydrolysis of industrial waste. Finally, the optimization of the physicochemical conditions for the enzymatic blends acting in the different types of industrial processes can lead to an increase in its efficiency, adding even more value to the desired product.

Declarations

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Conflicts of interests

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Yuri Heck da Silva, Tássio Brito de Oliveira and Rosymar Coutinho de Lucas. The first draft of the manuscript was written by Yuri Heck da Silva and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

All data generated or analysed during this study are included in this published article, and its supplementary information file.

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Tables

Table 1: Enzymatic profile of *A. brasiliensis*, *A.fumigatus var. niveus* and *Talaromyces sp* (120h of culture).

Substrate	Activity (U/mL)		
	A. brasiliensis	*A. fumigatus var. niveus*	*Talaromyces sp*
Xylan from beechwood	0.30	3.24	5.5
Xyloglucan	0.,06	0.37	0.9
CMC	0.04	0.05	0.08
pnp-β-D-cellobioside	0.004	0.01	0.02
β-glucosidase	0.03	0.09	0.03

Figures

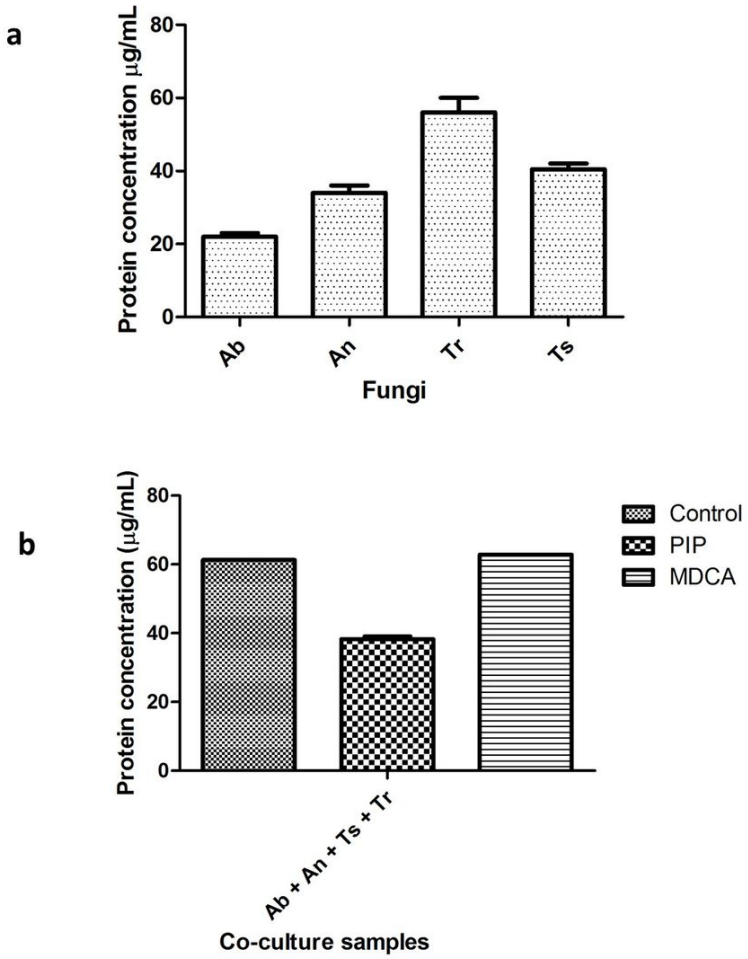

Figure 1

a Protein concentration of *A. brasiliensis*, *A. fumigatus var. niveus* and *Talaromyces sp* (120h of culture) grown individually on control bagasse. **b** Protein concentration of *A. brasiliensis*, *A. fumigatus var. niveus* and *Talaromyces sp* (120h of culture) grown in co-cultivation on control and pretreated bagasse.

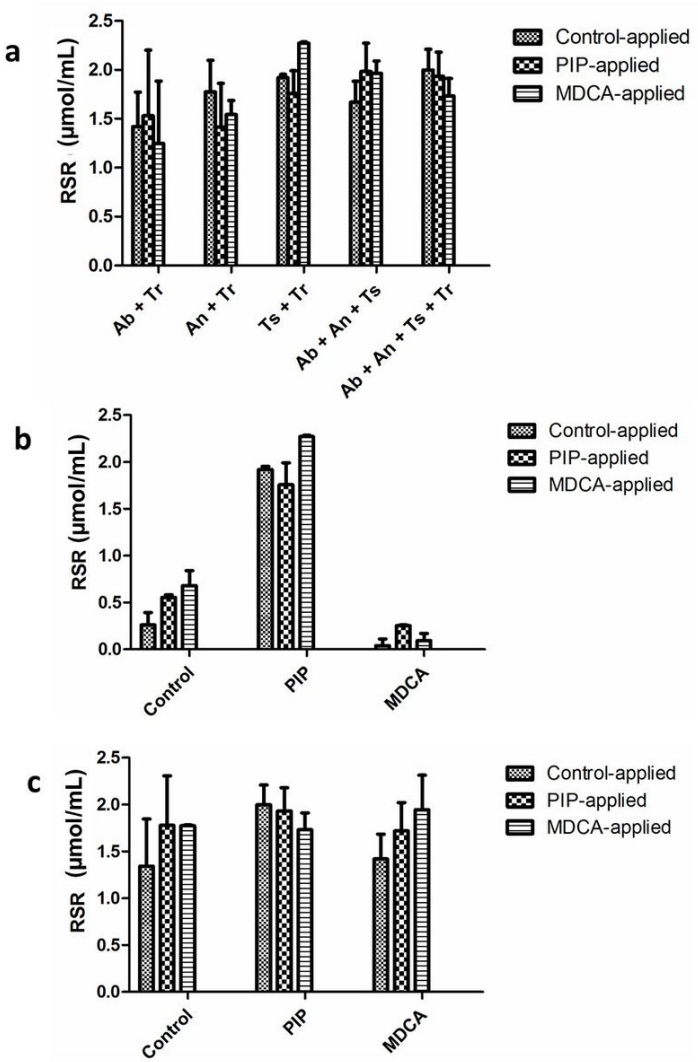

Figure 2

a Reducing sugars released in the hydrolysis of pretreated (PIP and MDCA) and control bagasse, using co-cultivation extracts produced in PIP bagasse. **b** Reducing sugars released using co-cultivation of *Talaromyces* sp. with *T. reesei* produced in PIP bagasse, MDCA and control. **c** Reducing sugars released using co-cultivation extracts of all fungi produced in PIP, MDCA and control bagasse. Ab– *A. brasiliensis*; An– *A. niveus*; Ts– *Talaromyces* sp.; Tr– *T. reesei*.

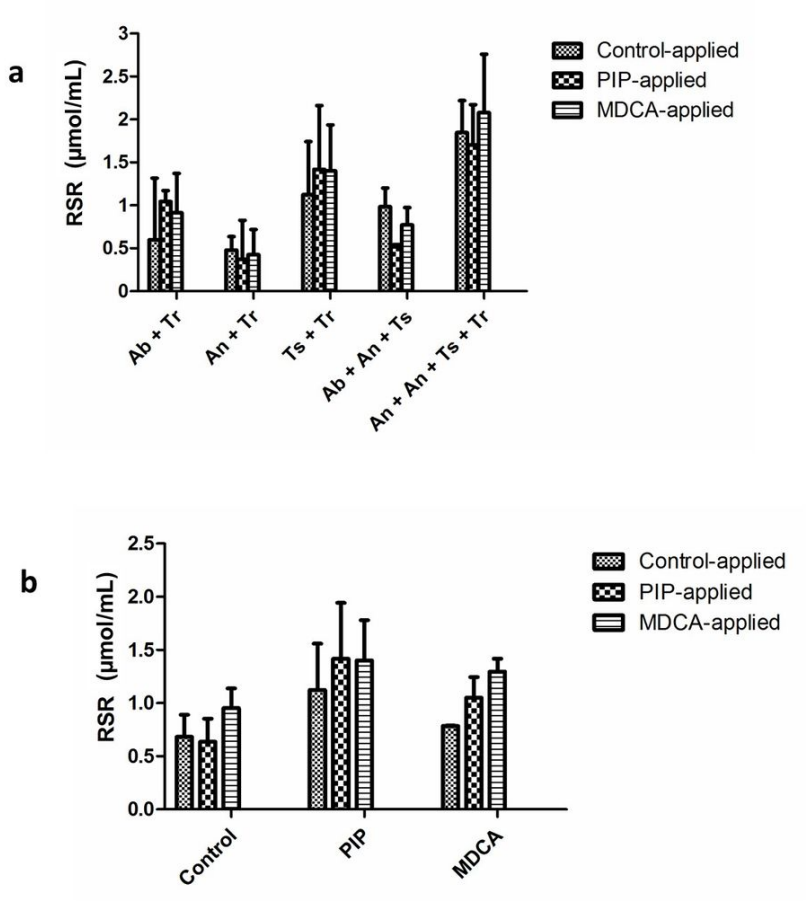

Figure 3

a Reducing sugars released in the hydrolysis of pretreated and control bagasse, using the combination of individual extracts produced in PIP bagasse. **b** Reducing sugars released using the combination of individual extracts of *Talaromyces* sp. and *T. reesei* produced in PIP bagasse, MDCA and control. Ab– *A. brasiliensis*; An– *A. niveus*; Ts– *Talaromyces* sp.; Tr– *T. reesei.*

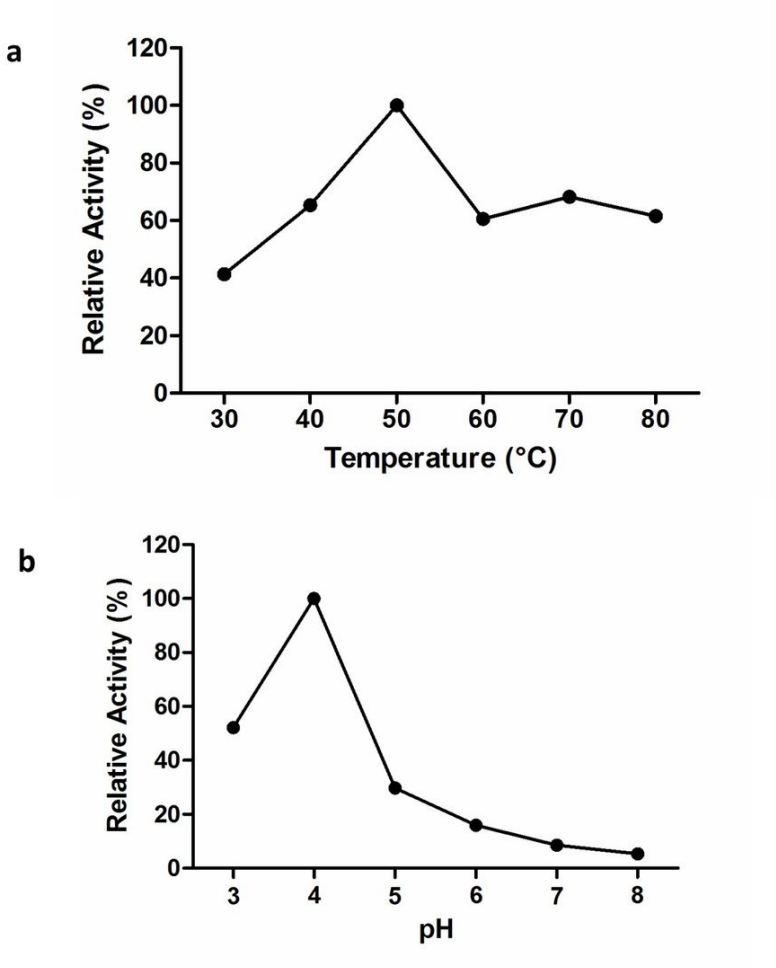

Figure 4

Best temperature and pH for the hydrolysis of control and pretreated bagasse, using the extract from the co-cultivation of all fungi from the cultivation in PIP bagasse.

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