Original Article

Biofpr
Biofuels Bioproducts & Biorefining

Colonization of oil palm empty fruit bunches by basidiomycetes from the Brazilian cerrado: deconstruction of biomass

Elias Alves da Silva, Programa de pós-graduação em Biotecnologia Vegetal, Universidade Federal de Lavras, Lavras, Brazil; Embrapa Agroenergia, Brasília, Brazil
Thais Demarchi Mendes, Thályta Fraga Pacheco, **Raquel Bombarda Campanha**, **Daiana Wischral**, **Simone Mendonça**, Embrapa Agroenergia, Brasília, Brazil
Marli Camassola, Instituto de Biotecnologia, Universidade de Caxias do Sul, Caxias do Sul, Brazil
Félix Gonçalves de Siqueira, Embrapa Agroenergia, Brasília, Brazil
Manoel Teixeira Souza Júnior, Programa de pós-graduação em Biotecnologia Vegetal, Universidade Federal de Lavras, Lavras, Brazil; Embrapa Agroenergia, Brasília, Brazil

Received March 09 2021; Revised August 31 2021; Accepted December 22 2021;
View online March 24, 2022 at Wiley Online Library (wileyonlinelibrary.com);
DOI: 10.1002/bbb.2339; *Biofuels, Bioprod. Bioref*. 16:799–815 (2022)

Abstract: The sustainable use of residual lignocellulosic biomass is an opportunity to help to overcome the global need to reduce the emission of pollutants as well as depletion of fossil fuel resources and increasing energy demands. This study aimed to establish a deconstruction process of oil palm empty fruit bunches (EFBs), employing hydrothermal and biological pretreatments. Initially, the yields of cellulose, hemicellulose, lignin, extractives and ashes resulting from the autohydrolysis of raw EFBs were measured. The biological pretreatment of the raw EFBs followed using eight basidiomycetes strains. Finally, an enzymatic hydrolysis comparison between basidiomycetes and commercial enzymes evaluated glucose and xylose yields, the synergism degree and the reduction of phenolic substances. Autohydrolysis pretreatment presented the best sugar yields after hydrolysis. However, biological pretreatment provides enzymes and other advantages. The combination of enzymatic extracts of basidiomycetes with Celluclast and Novozyme-188 Sigma® gave the best glucose yield with *Flavodon flavus* BRM-055676 (14.78%). Synergism degree analyses showed an increase of 47% in glucose release by the cocktail of *Fomes fasciatus* BRM-055675 with commercial enzymes. The deconstruction of EFBs by biological pretreatment presented a 2.96 ratio loss of lignin/loss of cellulose with *F. flavus* BRM-055676. Finally, combinations of enzymatic extracts from basidiomycetes and ascomycetes, mainly *F. fasciatus* BRM-055675, provided the reduction of phenolic substances. © 2021 Society of Chemical Industry and John Wiley & Sons, Ltd

Key words: biorefinery; basidiomycetes; biological pretreatment; enzymatic hydrolysis; delignification; *Elaeis* spp.

Correspondence to: Manoel Teixeira Souza Júnior or Félix Gonçalves de Siqueira, Brazilian Agricultural Research Corporation, Embrapa Agroenergy, Brasília, DF, Zip Code 70770-901, Brazil. E-mail: manoel.souza@embrapa.br; felix.siqueira@embrapa.br

Introduction

Lignocellulosic biomass can be converted to cellulose and hemicellulose polysaccharides and into fermentable or added-value chemical sugars in biorefineries. Lignin present in this material confers high recalcitrance, hindering enzymatic hydrolysis.[1] The palm oil industry would fit the biorefinery model because it generates millions of tons of lignocellulosic waste annually in the form of empty fruit bunches (EFBs), improper disposal of which could result in environmental damage.[2] According to Amelia *et al.*,[3] the oil palm sector generates about 25 million tons of EFBs each year, and the combustion processing of this residue to generate electricity releases carbon dioxide and nitrogen into the environment. These waste gases are harmful to the environment and can cause severe air pollution.

The use of physical, thermal, chemical and biological pretreatment methods, alone or combined, is indispensable to break the lignocellulosic structures and facilitate subsequent enzymatic hydrolysis. The goal is to deconstruct the cell wall and rupture polysaccharide chains, which is necessary to enhance the accessibility of enzymes during enzymatic hydrolysis to fermentable sugar (glucose and xylose).[4,5] All of the methods have advantages and disadvantages; however, chemical and physical ones that employ high temperatures end up leading to pollutants and the formation of inhibitors for hydrolysis and fermentation.[6]

The use of biological pretreatments, mainly using macro-basidiomycetes, has been presented as a promising alternative since these fungi are enzyme producers capable of modifying lignin,[7] bio-detoxifying biomass minimizing the effect of inhibitors from pretreatment and improving biomass biodegradability.[8] The use of biological pretreatments requires organisms to improve the biomass digestibility and act selectively on lignin, preserving the maximum cellulose and hemicellulose polysaccharides.[5]

Macro-basidiomycetes present an enzyme apparatus that is rich in lignocellulolytic enzymes, making them the significant decomposition agents of plant cell walls in nature.[7] However, the long time needed for colonization of these fungi remains a disadvantage of this process. The combination of fungal and chemical or physical pretreatments could maximize glucose yield and reduce possible inhibitors. The selectivity of some basidiomycetes in lignin degradation stands as one of the advantages of these processes.[5,9,10] Some studies have suggested mixing crude enzymatic extracts of basidiomycetes into cocktails to verify the synergistic effects and possibly reduce the amount of commercial enzymes (CEs) applied in hydrolysis processes.[11,12]

The present study aimed to evaluate enzymatic hydrolyses of EFBs with enzymatic extracts obtained from basidiomycetes and ascomycetes, alone or in combination with commercial cellulases, and characterize the release of sugars and the removal of inhibitors.

Materials and methods

Hydrothermal pretreatment of empty fruit bunches

The EFBs and sludge from the palm oil (*Elaeis* spp.) decanter (SD) were provided by DENPASA (Dendê do Pará S/A). EFB biomass was subjected to hydrothermal pretreatment (autohydrolysis) in a Parr reactor (4520, Parr Instruments Company, Moline, Illinois, USA). Aliquots of 60 g of EFBs and 710 mL of tap water were mixed, and the mixture was subjected to heating at 180 °C for 40 min and constant stirring at 600 rpm. After cooling, EFBs were subjected to simple filtration in gauze for liquor removal. Then, the now-called hydrothermally pretreated bunch (EFB-AH) had its moisture content determined and stored in a closed container at 4 °C.

Biological pretreatment of EFBs by macro-basidiomycetes

The biomasses (10 g of EFBs, 80 mesh) were placed in 250 mL Erlenmeyer flasks for solid-state cultivation (SSC). The humidity was adjusted to 65–70% with the addition of distilled water, followed by sterilization for 30 min at 121 °C and overpressured 1 atm. Eight strains were cultured for enzyme production: *Flavodon flavus* (BRM-055676), BRM-063103, *Fomes fasciatus* (BRM-055675), *Pleurotus* sp. (BRM-062379), *Trametes* sp. (BRM-060007), *Pycnoporus* sp. (BRM-062381), *Coprinus* sp. (BRM-050072) and *Pleurotus* sp. (BRM-060012), cultivated for 21 days at 28 °C. The crude enzyme extracts (CEEs) were obtained by washing the colonized biomass with a solution of Triton X-100 (0.1%), in a 1:10 ratio (w/v), using a shaker under stirring at 200 rpm, at 5.0 °C for 40 min. Then the extract was filtered with gauze and centrifuged at 10 600×*g* and 4.0 °C for 10 min, and the supernatant was collected. Sodium azide (final concentration of 0.02%) was added to the CEEs to prevent the growth of contaminating microorganisms. The enzymatic extract was stored at 4.0 °C.

After macro-basidiomycetes culture in Erlenmeyer flasks with EFBs, the lignocellulosic biomasses resulting were named biologically pretreated biomasses (BEB). They correspond to a mixture of plant biomass and microbial mass. These BEBs were lyophilized for 48 h and crushed (2 mm) in a bench mill (IKA 11®). Percentages of mass losses for EFBs after biological pretreatment were calculated.

Production of crude enzyme extracts by ascomycetes

The references used for cellulolytic extract production were the ascomycetes *Trichoderma reesei* (ATCC60787) and *Aspergillus aculeatus* (F-50 NBRC108796). These ascomycetes were grown in a sterile liquid culture medium, autoclaved at 121 °C for 30 min at 1 atm,[13] and supplemented with 2.5% SD (80 mesh) to obtain the CEEs. Six 7 mm mycelial disks of *T. reesei* and *A. aculeatus*, previously cultivated individually in potato dextrose broth (PDA) for 7 days, were used as inoculum for liquid cultures. The flasks were incubated for 7 days in a shaker at 28 °C and 150 rpm. Samples of 1 mL were collected daily to check the enzyme profile and soluble protein content. At the end of the cultivation period, it was centrifuged at 10 600×*g*, at 4.0 °C, for 10 min to obtain the CEEs. The CEEs were then kept at 4.0 °C until used in the cocktail formulation for enzymatic hydrolysis.

Characterization of biomass

The different types of biomass (EFBs, SD, EFB-AH and BEB) were characterized for their contents of cellulose (glucan), hemicellulose (sum of arabinan, galactan, xylan, mannan and acetyl), lignin (fractionated into insoluble acid material and soluble acid material), extractives and ashes, according to methodologies recommended by the National Renewable Energy Laboratory, Golden, CO, USA.[14–16] The coefficients of molar absorptivity of lignin from the lignocellulosic substrates were determined (2.2146 L g^{-1} cm^{-1} at 320 nm). Also, the SLC (loss of lignin/loss of cellulose ratio) and SLH (loss of lignin/loss of holocellulose ratio cellulose + hemicellulose) parameters were calculated.

Enzymatic hydrolysis of EFB-AH

The enzymatic hydrolyses were performed in 24 deep-well plates, with a solid load of 5% (dry matter) biomass added to the buffer solution (sodium citrate/citric acid, 100 mmol dm^{-3}, pH 5.0), to a final volume of 2.2 mL. All of the experiments were carried out in triplicate. The hydrolysis plates were sealed and kept in a shaker at 50 °C and 200 rpm for 24 h. Samples were collected and centrifuged at 10 600×*g*, at 4.0 °C, for 10 min, and the supernatant was stored at −20 °C.

First, the CEEs of two macro-basidiomycetes (*F. flavus* BRM-055676 or *F. fasciatus* BRM-055675) were lyophilized and resuspended in distilled water, and concentrated five times (CEEc5). The following mixtures of CEEc5 of basidiomycetes (12.69 mL g^{-1} dry weight) were prepared: concentrated five times (A) and 2.5 times (B), diluted five times (C) and not concentrated (D). Then, the hydrolyses of EFB-AH were carried out with two different enzyme mixtures. Mixture 1 comprised CEEs of macro-basidiomycetes with concentrations A–D (12.69 mL g^{-1} of EFB-AH dry weight) with CE cellulase of *T. reesei* (ATCC 26921; Celluclast®; Sigma-Aldrich®) and cellobiase of *Aspergillus niger* (Novozyme-188®; Sigma-Aldrich®) and the following protein dosages: 8.125 and 4.375 mg g^{-1} dry weight. Mixture 2 comprised CEEs of basidiomycetes with concentrations A–D (12.69 mg g^{-1} of EFB-AH dry weight) and CEEs of *T. reesei* and *A. aculeatus* ascomycetes, with protein dosages of 8.128 and 4.375 mg g^{-1} dry weight, respectively.

Synergism

The synergism degree allows us to verify whether there was cooperation between the different enzymes concerning the release of glucose and xylose during the enzymatic hydrolysis of EFB-AH. The synergism degrees of these hydrolyses are based on the quantification of sugars. The synergism degree was calculated according to Eqn (1), as described by Arias *et al.*,[17] evaluating the sugar yields of each extract (CEEs of macro-basidiomycetes and ascomycetes and commercials) when compared with those of the mixtures.

$$\text{Synergism degree} = \frac{\text{Glucose released}\left(\text{mixture of } n \text{ components}\right)}{\sum_{i=1}^{i=n} \text{glucose released for each component } i} \quad (1)$$

Enzymatic hydrolysis of BEFB

Saccharification through enzymatic hydrolysis of BEFB with CEs was carried out with Cellic® CTec3® (Novozymes). Experiments were prepared in 2 mL Eppendorf tubes with BEFB containing extractives (samples without washing) and free of extractives (BEB subjected to an accelerated extraction using water/ethanol solvents; Dionex™ ASE™ Thermo Fisher Scientific, Waltham, MA, USA; 5% solids content). Experiments with EFBs (untreated) were performed in parallel as a control. To each tube was added 1.40 mL of the enzyme (15 FPU g^{-1} dry weight) diluted in citrate buffer (0.1 mol dm^{-3} sodium citrate and 0.1 mol dm^{-3} citric acid) pH 5.0, and sealed with parafilm 'M' Kasvi® and then placed in a thermoblock (Eppendorf ThermoMixer® C) at 50 °C and 800 rpm for 24 h. All experiments were done in triplicate. At the end of enzymatic hydrolysis, the tubes were centrifuged at 10 600×*g*, at 4.0 °C, for 10 min. Supernatants were then collected for sugar quantification, and all experiments were done in triplicate.

Enzymatic hydrolysis of BEB pretreated by autohydrolysis

Enzymatic hydrolyses of biomasses pretreated by combined biological and hydrothermal treatments were carried out in 24 well-deep plates at pH 5.0 and 50 °C for 24 h. The solids load was 5% (dry matter), and the final volume of the enzyme was standardized at 2.6 mL. EFB hydrolyses were evaluated as: (i) raw EFBs; (ii) EFBs pretreated with *F. flavus* BRM-055676; (iii) EFBs pretreated by autohydrolysis (EFB-AH); and (iv) EFBs pretreated with *F. flavus* BRM-055676 followed by autohydrolysis. The enzymatic cocktails applied were: (i) Celic CTec3 (Novozymes; 15 FPU g^{-1} dry weight) as control; (ii) (Tr + Aa) (12.5 mg g^{-1} dry weight); (iii) *F. flavus* BRM-055676 (12.69 mL g^{-1} dry weight) + (Tr + Aa) (12.5 mg g^{-1} dry weight); or (iv) *F. fasciatus* BRM-055675 (12.69 mL g^{-1} dry weight) + (Tr + Aa) (12.5 mg g^{-1} dry weight). The enzymatic load of basidiomycetes during hydrolysis was standardized in a volume since the protein concentration in the crude extracts varies according to the species and is not directly proportional to the respective enzymatic activity, and basidiomycetes extracts have shown enzymes acting in lignin (laccases and peroxidases). Supernatants were collected for sugar quantification, and all experiments were done in triplicate.

Removal of enzymatic inhibitors

The potential for removing phenolic compounds and inhibitors by the action of enzymes was measured through experiments carried out with CEEs of macro-basidiomycetes alone and combined with CEEs of ascomycetes *T. reesei* and *A. aculeatus* (Tr + Aa). These experiments were performed under the same conditions of hydrolysis but without lignocellulosic biomass. A mix of pattern chemical substances was formulated with gallic acid, vanillic acid, hydroxymethylfurfural (HMF), furfural, vanillin, cumaric acid, syringaldehyde, ferulic acid and synaptic acid (Sigma-Aldrich), for a final concentration of 1 g L^{-1}, mixed with sodium citrate/citric acid buffer 0.1 mol dm^{-3} (pH 5.0) and CEEs. The deep-well plates were sealed and incubated at 50 °C in a shaker at 200 rpm for 180 min. Samples of 1 mL were taken and frozen for posterior quantification of sugars. The removal percentage was determined based on the controls (standards substances without CEEs), and all experiments were done in triplicate.

Analytical methods

Enzymatic loads were standardized by determining the FPase activity followed the colorimetric method with miniaturized DNS, proposed by Xiao *et al.*[18] The content of total soluble proteins in the crude extracts was determined through the bicinchoninic acid method[19] in ELISA plates according to the protocol Sigma-Aldrich® commercial kit. Values were converted to mg g^{-1} of the lignocellulosic substrate, taking into account the volume of the extraction solution (Triton X-100 0.1%) and the amount of substrate used in the system.

Quantification of glucose and xylose, after enzymatic hydrolysis, was performed using high-performance liquid chromatography (HPLC; Agilent Technologies), under the following conditions: Aminex® column HPX-87H (300 × 7.8 mm, Bio-Rad, solution of H_2SO_4 (5 mmol dm^{-3}) as the mobile phase; column temperature at 45 °C, running time of 12 min and a flow rate of 0.6 mL min^{-1}. The hydrolysis yields were determined compared with the theoretical maximum.[12] HPLC was used to quantify lactic acid, succinic acid, levulinic acid, acetic acid and formic acid. Chromatographic conditions were the following: refractive index detector, Aminex HPX-87H column with pre-column, injection volume of 10 µL; mobile phase composed of 5 mmol dm^{-3} H_2SO_4 solution; flow rate of 0.6 mL min^{-1}; column temperature at 45 °C; and detector temperature at 45 °C.

For quantification of gallic acid, vanillic acid, HMF, furfural and vanillin, using a diode array detector, an Acquity UPLC HSS T3 1.8 µm 2.1 × 150 mm column was used with a pre-column. Detections were at 280 nm and 320 nm (coumaric acid, syringaldehyde, ferulic acid and synaptic acid), with an injection volume of 1 µL. Mobile phase A was 0.1% formic acid and mobile phase B was acetonitrile. The following gradient was used: 0 min (90% A and 10% B), 5 min (80% A and 20% B), 7.5 min (75% A and 25% B) and 12.5 min (55% A and 45% B). The workflow was 0.4 mL min^{-1} and the column temperature was 40°C.

Results and discussion

Characterization of biomass pretreated by autohydrolysis

The compositions of cellulose, hemicellulose, lignin and extracts were determined from raw and pretreated lignocellulosic material by autohydrolysis (Table 1). In absolute values, EFBs present a higher amount of polysaccharides than SD. Lignin represents a considerable fraction of the residues, and the increase in its concentration in EFB-AH may be due to the removal of hemicellulose in autohydrolysis. The protein amounts close the mass balance, presenting 2.08 ± 0.12% from empty fruit bunches and 15.44 ± 0.17% from sludge from the oil decanter.

EFBs represent a promising biomass for obtaining fermentable sugars; however, without any pretreatment,

Table 1. Chemical composition of some of the residues from palm oil agroindustry.

Component	EFBs (%)	SD (%)	EFB-AH (%)
Lignin	27.69 ± 0.41	25.74 ± 0.35	42.10 ± 0.27
Cellulose	31.15 ± 0.53	18.22 ± 0.73	38.59 ± 1.80
Hemicellulose	16.37 ± 0.21	12.39 ± 0.19	8.75 ± 0.33
Extractives	14.91 ± 0.29	21.38 ± 0.07	Not applicable
Ashes	5.99 ± 0.02	7.56 ± 0.06	0.57 ± 0.14
Acetyl	3.49 ± 0.03	1.61 ± 0.01	0.65 ± 0.02

EFBs, Empty fruit bunches; SD, sludge from oil decanter; EFB-AH, empty fruit bunches pretreated by autohydrolysis.

yields of reducing sugars are limited (0.4–2.6%). The presence of lignin and hemicellulose in this material is the reason for lower sugar production.[20] Corroborating this, it can be observed that, under the conditions tested, the hydrothermal pretreatment mainly hydrolyzed EFB hemicellulose. Thamvithayakorn *et al.*[21] characterized oil palm decanter cake that presented 30.62% lignin, 14.71% hemicellulose and 22.39% cellulose. Abdul *et al.*[2] studied oil palm EFB composition and achieved 50.5% cellulose, 29.6% hemicellulose, 17.8% lignin, 3.4% ash and 3.2% extractive. Jung *et al.*[22] performed pretreatment of empty palm fruit bunches (5%, w/v) with 1 mol dm^{-3} NaOH at 121 °C, and the characterization after pretreatment showed a composition of 58% cellulose, 21.1% hemicelluloses, 8.8% lignin, 8.9% extractives and 3.2% ash.

Characterization of biomass pretreated by macro-basidiomycetes

Regarding lignin, treatments used traditionally in the industrial process of obtaining the oil and the one used in this study (hydrothermal) may have contributed to the collapse of the EFB lignin. The loss of structural components of biomass was quantified after pretreatm with basidiomycete strains (BEFB), as shown in Fig. 1(A–E). One can also observe that in the analysis of the characterization of lignocellulosic biomass, mass closure of around 100% was obtained (Fig. 1(E)).

The results show that the tested macro-basidiomycete strains can metabolize cellulose, hemicellulose, lignin and extractives as carbon and nitrogen sources, with the loss of mass (Fig. 1(A)). Such losses are expected since fungi convert part of the carbon/nitrogen sources present in plant biomass into fungal biomass (mycelium), that is, a loss owing to cellular respiration, releasing CO_2 and water. The highest losses of mass observed occurred for *Trametes* sp. BRM-060007 and *Pycnoporus* sp. BRM-062381 and then for *F. flavus* BRM-055676.

The highest losses of cellulose (glucan) occurred in the cultivation of *Pycnoporus* sp. BRM-062381, and then *Trametes* sp. BRM-060007, *Coprinus* sp. BRM-050072 and *Pleurotus* sp. BRM-060012 (Fig. 1(B)). Concerning hemicellulose, the highest losses occurred for *Pleurotus* sp. BRM-062379 and BRM-060012 (Fig. 1(C)), while delignification was higher for *F. flavus* BRM-055676 and *F. fasciatus* BRM-055675 (Fig. 1(A–E)).

The strains BRM-060012, BRM-062379 and BRM-063103 resulted in the highest levels of extractives loss – fatty acids, simple sugars, waxes and sterols (Fig. 1(E)). The carbon sources present in the holocellulose (cellulose and hemicellulose) are generally better metabolized than lignin. Biological treatments with macro-basidiomycetes during longer cultivation times (30, 45, 60 or more days) may show more significant de-lignification results for some species;[6,7] however, with higher losses of holocellulose (carbohydrates).

Nevertheless, the main goal of many studies, including this one, is to obtain fungal strains that could delignify biomasses in solid-state cultivation in a shorter time, leading to higher levels of sugars and the absence of enzymatic or fermentative inhibitors. Degradation of lignin by macrofungi is related to variations in the consumption of sugars, obtained from the breakdown of holocellulose, for the fungal growth itself.[9] The preference of some fungi for hemicellulose has been attributed to its lower degree of polymerization.[23] In the case of the EFBs used, hemicellulose represents 16.37%, while cellulose represents 31.15%. The results of this study indicated that the tested strains were capable of simultaneously degrading these polysaccharides.

Macro-basidiomycetes make use of their enzyme apparatus to break down the lignin polymer to alter the structure of lignocellulose.[5,7] Selectivity of these fungi for this polymer is obtained based on the ratio of loss of lignin to loss of cellulose (SLC). In this way, the best fungi for application in biological pretreatment are those that promote a high reduction of lignin and less consumption of sugars (holocellulose or cellulose) in a shorter time.[9]

The selectivity of macro-basidiomycete in degrading more lignin than cellulose (SLC) of EFBs was one criterion for selecting the best candidate to supply pretreated biomass with a higher potential for further sugar release. Under the conditions tested, the species that best showed this selectivity was *F. flavus* BRM-055676 (2.96), followed by BRM-063103 (nd) (2.17) and *F. fasciatus* BRM-055675 (1.01), while the other strains showed selectivity lower than 1.0, indicating a higher consumption of cellulose concerning the lignin contained in the EFBs of palm oil (Table 2).

In addition, a second selectivity was obtained, which consisted of the loss of lignin/loss of holocellulose ratio (sum

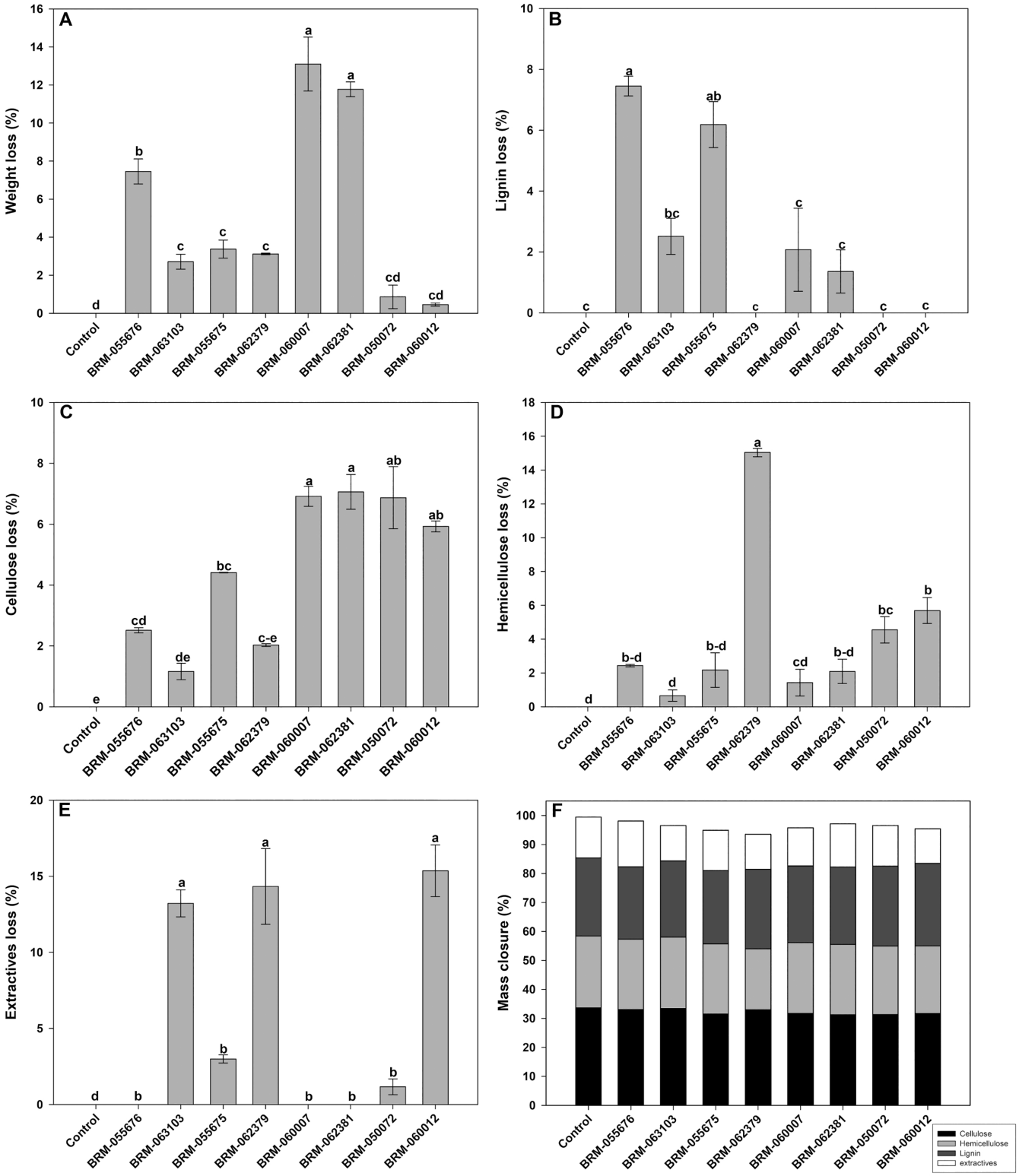


Figure 1. Composition of mass and structural components (%) of the empty bunches pretreated with macro-basidiomycetes (BEB) in solid-state cultivation for 15 days at 28 °C and 65% (±5) of moisture. Weight loss (A), lignin (B), cellulose (C), hemicellulose (D), extractives (E) and mass closure (F). Data presented represent the mean and standard error of an experiment carried out in triplicate. Different letters indicate statistically significant differences using the Tukey test ($P < 0.05$).

Table 2. Loss of structural components (%) after biological pretreatment with macro-basidiomycetes (BEB) for 15 days at 28 °C and 65% (± 5) of moisture level.

Strain*	Lignin (%)	Hemicellulose (%)	Cellulose (%)	SLC	SLH
Flavodon flavus BRM-055676	7.45 ± 0.56^{a}	2.43 ± 0.12^{a}	2.51 ± 0.14^{c}	2.96^{a}	1.75^{a}
BRM-063103	2.51 ± 1.03^{bc}	1.16 ± 0.6^{a}	1.16 ± 0.26^{c}	2.17^{ab}	1.64^{a}
Fomes fasciatus BRM-055675	6.19 ± 1.31^{ab}	2.17 ± 1.76^{a}	4.41 ± 0.13^{b}	1.40^{bc}	1.01^{ab}
Trametes sp. BRM-060007	2.07 ± 2.37^{bc}	1.43 ± 1.36^{a}	6.91 ± 0.57^{a}	0.24^{c}	0.2^{b}
Pycnoporus sp. BRM-062381	1.36 ± 1.23^{c}	2.09 ± 1.24^{a}	7.06 ± 0.98^{a}	0.17^{c}	0.2^{b}

Data shown are the means ± standard deviation for experiments performed in triplicate. Different letters in the column indicate statistically significant differences using the Tukey test ($P < 0.05$).
SLC, Ratio between loss of lignin and loss of cellulose; SLH, ratio between loss of lignin and loss of holocellulose (cellulose + hemicellulose).
*Strains that showed negative selectivity were omitted from the table.

of the components cellulose and hemicellulose), which was called Lignin-Selectivity/Holocellulose – SLH. Based on this selectivity, there are no statistically significant differences between the strains tested; however, the most selective ones (lignin/cellulose) presented values of SLH >1.0, indicating higher lignin losses concerning cellulose and hemicellulose polysaccharides (Table 2).

Other studies involving biological pretreatment of biomasses resulted in different selective fungi for lignin. The lignin/cellulose selectivity criterion is usually employed to select biological pretreatment agents for plant biomass. Partial de-lignification of biomass is a crucial step to obtain pretreated biomass. The de-lignified biomass is consequently easily deconstructed in the enzymatic hydrolysis step, releasing higher concentrations of soluble sugars and minimizing the formation of enzymatic and fermentative inhibitors, which usually come from lignin.

The basidiomycete *Trametes versicolor* showed an SLC of 1.57 after SSC for 14 days, using EFBs as a substrate.[24] In another study, now using fungus *Irpex lacteus* Fr.238617/93 grown in lignocellulosic residues (corn straw, barley straw, corn cob and wheat straw), after 21 days of colonization, the SLCs were 1.38, 2.10, 1.54 and 1.40, for each biomass tested, respectively.[9]

The biological pretreatment of corn stover, with 26 macro-basidiomycetes in the SSC system for 30 days indicated that *Phlebia brevispora* NRRL-13108 was the strain (SLC 2.46) that was most selective.[7,10] In another study, white-rot fungus *Phanerochaete chrysosporium* (ATCC 24725) presented an SLC of 1.83 during 15 days of cultivation in the SSC system, using rapeseed straw as a substrate.[25]

For the biological pretreatment of plant biomass, choosing the macro-basidiomycete species is extremely important to obtain total reducing sugars. However, other experimental variables are also truly relevant, such as the type of biomass and cultivation conditions (humidity, temperature and incubation time), which can even influence SLC results. In addition, species used in these studies may behave differently owing to the differential expression of genes that encode ligninolytic and cellulolytic enzymes in response to variations in these conditions.[26]

Enzymatic hydrolysis of EFB-AH

A positive effect on the release of sugars was verified using CEEs of *F. flavus* BRM-055676 or *F. fasciatus* BRM-055675 without any concentration step (12.69 mL g^{-1} of EFB-AH dry weight), corresponding to 1.2 mL of CEE extract volume. As it was impossible to increase the amount of CEEs owing to the limitation of the reaction volume, the extracts were concentrated five times before adding to the enzymatic mixtures to verify improvements in the yields of EFB-AH. In this step, the *F. flavus* BRM-055676 and *F. fasciatus* BRM-055675 extracts did not undergo any purification and separation before the concentration stage.

The results of the hydrolysis with concentrated CEEs of *F. flavus* BRM-055676 and *F. fasciatus* BRM-055675 with CEEs of the ascomycetes *T. reesei* and *A. aculeatus* and CEs are provided in Fig. 2. In general, a positive effect appeared when combining *F. flavus* BRM-055676 or *F. fasciatus* BRM-055675 with the CE: Celluclast and Novozymes-188. In comparison with the control (only CE), the highest glucose yield occurred for CE + *F. flavus* BRM-055676 (C) (Fig. 2(A)), while in terms of xylose yield, no significant difference was verified (Fig. 2(B)). Glucose releasing reached (3.17 ± 0.13) g L^{-1}, which corresponds to a 14.78 ± 0.60% yield.

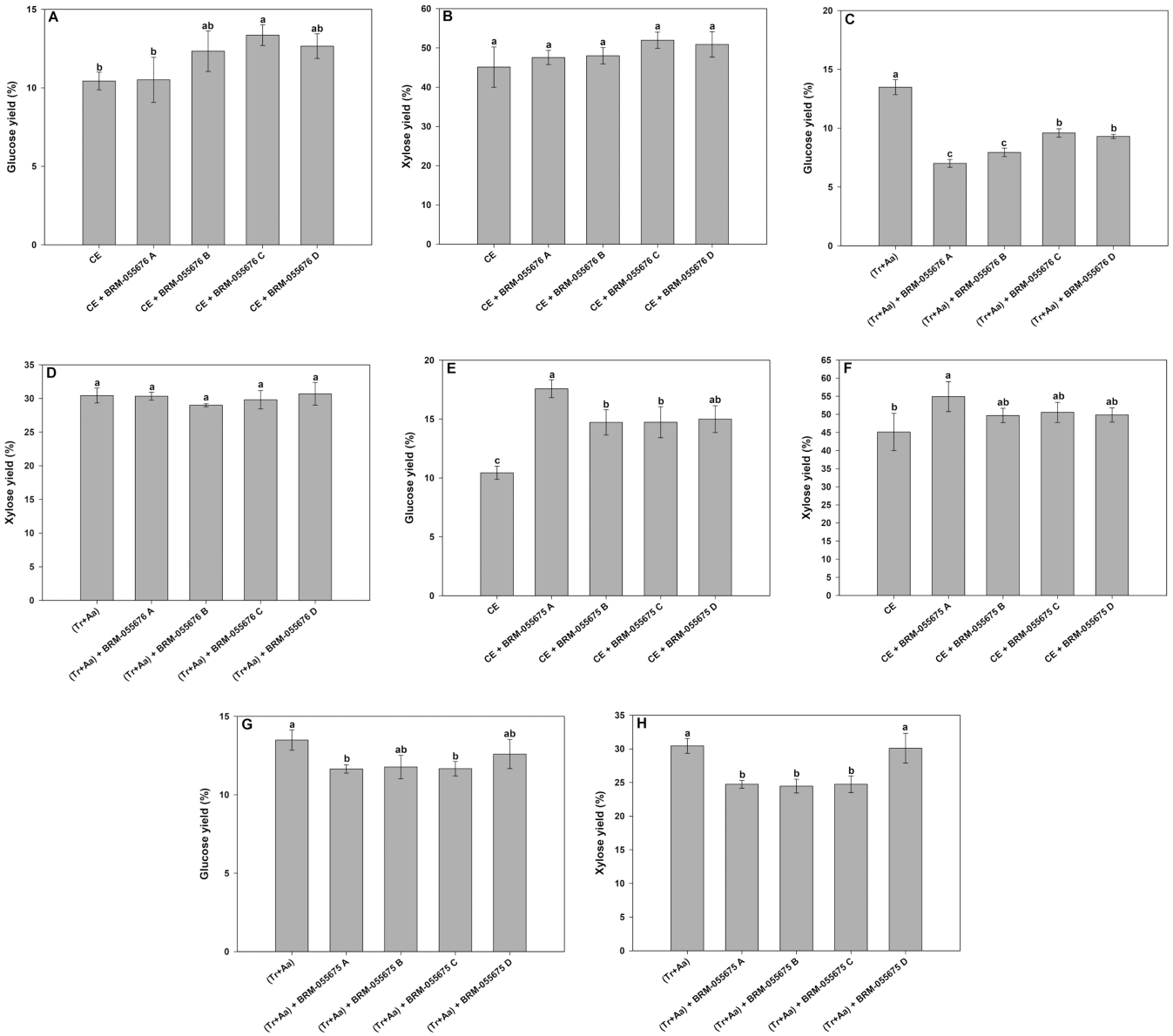


Figure 2. Yield (%) of glucose and xylose after 24 h hydrolysis of EB-AH, at pH 5.0 and 50 °C, applying different enzymatic extractants: (A, B) with CE and *Flavodon flavus* BRM-055676; C-D with (Tr + Aa) and *F. flavus* BRM-055676; (E, F) with CE and *Fomes fasciatus* BRM-055675; (G, H) with (Tr + Aa) and/or *F. fasciatus* BRM-055675. Enzymatic extracts of basidiomycetes *F. flavus* BRM-055676 and *F. fasciatus* BRM-055675 (12.69 mL g^{-1} dry weight) were prepared: concentrated 5 times (A); concentrated 2.5 times (B); diluted 5 times (C); and no concentration (D). CE extract = cellulase (8.125 mg g^{-1} dry weight) and cellobiase (4.375 mg g^{-1} dry weight) (Sigma-Aldrich); (Tr + Aa) extract = *Trichoderma reesei* (8.125 mg g^{-1} dry weight) and *Aspergillus aculeatus* (4.375 mg g^{-1} dry weight). Data shown represent the average of analyses performed in triplicate for each essay also performed in triplicate. Different letters indicate statistically significant differences using the Tukey test ($P < 0.05$).

The concentrated extract of *F. fasciatus* BRM-055675 (A) improved the glucose and xylose yields compared with the respective controls (Fig. 2(E, F)). For the cocktail with ascomycetes extracts, there was no positive effect of the combinations (Tr + Aa) + *F. flavus* BRM-055676 (Fig. 2(C, D)), while for *F. fasciatus* BRM-055675, the results were close to those of the control in terms of xylose and glucose yields, at the tested conditions (D) (Fig. 2(G, H)).

When comparing efficiency between the CEEs without lyophilizing (Fig. 2(A–H), concentration C) and the CEEs lyophilized once (Fig. 2(A–H), concentration D), the lyophilization procedure did not affect the synergism of the

CEEs from macro-basidiomycetes in combination with the enzymes Celluclast and Novozymes-188, leading to yields close to those previously achieved.

The concentration of enzymes to use in hydrolysis must be studied to reduce it without compromising the yield of fermentable sugars. De La Torre *et al.*[27] used the lowest possible concentration of the CEs Celluclast 1.5, Novozyme-188 and Pectinex Ultra SP in cocktails during the hydrolysis of orange peel residues and observed that reductions in concentration resulted in a lower initial reaction rate and a lower yield. Moreover, the saccharification process took place more slowly.[27] A pre-optimization study concerning saccharification of sugarcane bagasse, Cellic CTec2 enzyme (Novozymes), led to a higher yield of conversion from cellulose to glucose when used in low enzymatic load and for high content of total solids.[28]

Cellulases and auxiliary enzymes tend to adsorb on lignocellulosic surfaces, creating areas with a high protein concentration and causing a reduction in average activities by overcrowding or interference, leading to a significant decrease in hydrolysis and consequently in glucose release[29] (Fig. 1(C)). However, the addition of non-enzymatic proteins, such as bovine serum albumin, in a simultaneous saccharification and fermentation system increased the conversion of cellulose and xylose and the yield of ethanol from rice straw.[30]

It is necessary to focus on the selection of potential fungi for the development of efficient cocktails. Additional strategies include metabolic engineering and stress engineering to improve enzymatic production. These approaches achieved many advances in recent years by applying emerging tools for genome editing based on CRISPR-Cas9 and synthetic biology.[31] Moreover, basidiomycetes are also sources of genes of industrial interest.[32]

Synergism

Synergism is the cooperation between enzymes of the same enzyme complex, and it depends on the proportions and characteristics of the enzyme and the substrate.[1,33]

Table 3 shows the synergism values for glucose and xylose, calculated for mixtures of CEs with CEEs of *F. flavus* BRM-055676 and *F. fasciatus* BRM-055675, and (Tr + Aa) with CEEs of *F. flavus* BRM-055676 and *F. fasciatus* BRM-055675 (calculated with results from Fig. 2(A–H)). The degree of synergism for glucose release ranged from 0.51 to 1.47, and for xylose release from 0.57 to 1.07. All extracts mixed with CEs had a degree of synergism for glucose >1, indicating a synergistic effect for glucose release. As for the release of xylose, only one of the extracts showed a degree of synergism >1, indicating that none of the mixtures had a synergistic effect for xylose release.

The treatment that showed the highest degree of synergism for both sugars was the mixture of the commercial extract with *F. fasciatus* BRM-055675 (concentration A: 5 times concentrated), with 1.47 and 1.07 for glucose and xylose, respectively. For *F. flavus* BRM-055676, the highest degree of synergism was observed with crude extract diluted five times (C), reaching 1.28 for glucose and 0.87 for xylose. Furthermore, according to the Tukey test ($P < 0.05$), there was no statistically significant difference between the different concentrations and dilution methods used with mixtures with the commercial extract.

Table 3. Synergism degree between CEEs of *Flavodon flavus* BRM-055676 or *Fomes fasciatus* BRM-055675 (different concentrations) and CEEs of (Tr + aa) or CE.

CEEs	Sugar	Synergism degree			
		A	B	C	D
CE + *F. flavus* BRM-055676	Glucose	1.01 ± 0.14[a]	1.18 ± 0.12[a]	1.28 ± 0.06[a]	1.21 ± 0.07[a]
	Xylose	0.79 ± 0.03[a]	0.80 ± 0.03[a]	0.87 ± 0.03[a]	0.85 ± 0.05[a]
CE + *F. fasciatus* BRM-055675	Glucose	1.47 ± 0.06[a]	1.23 ± 0.09[a]	1.23 ± 0.11[a]	1.25 ± 0.10[a]
	Xylose	1.07 ± 0.08[a]	0.97 ± 0.04[a]	0.99 ± 0.05[a]	0.97 ± 0.04[a]
(Tr + Aa) + *F. flavus* BRM-055676	Glucose	0.51 ± 0.02[c]	0.58 ± 0.03[b]	0.70 ± 0.03[a]	0.68 ± 0.01[a]
	Xylose	0.79 ± 0.02[a]	0.75 ± 0.01[a]	0.77 ± 0.04[a]	0.80 ± 0.04[a]
(Tr + Aa) + *F. fasciatus* BRM-055675	Glucose	0.68 ± 0.02[a]	0.69 ± 0.04[a]	0.68 ± 0.03[a]	0.74 ± 0.05[a]
	Xylose	0.58 ± 0.01[b]	0.57 ± 0.02[b]	0.58 ± 0.03[b]	0.70 ± 0.05[a]

Enzymatic hydrolyses were performed with EFB-AH at pH 5.0, 50 °C, during 24 h. A, CEEs concentrated 5 times; B, CEEs concentrated 2.5 times; C, CEEs diluted 5 times; and D, CEEs. Combination of CE with cellulase (0.65% with 12.5 mg g^{-1}) and cellobiase (0.35% with 12.5 mg g^{-1}) and enzymatic charge of *F. flavus* BRM-055676 and *F. fasciatus* BRM-055675 was 12.69 mL g^{-1}. Different letters in the column indicate statistically significant differences using the Tukey test ($P < 0.05$).

Differences concerning sugar release from extracts *F. flavus* BRM-055676 and *F. fasciatus* BRM-055675 were probably due to the different enzymatic compositions of these extracts, as reported in Fig. 1. In addition to cellulases and xylanases, *F. fasciatus* BRM-055675 also presented lignin peroxidase, which hydrolyzes lignin, possibly reducing the recalcitrance of this biomass (caused by lignin) and favoring the action of cellulases and xylanases. The results from Fig. 1(B) confirmed this hypothesis, presenting a lower lignin concentration in *F. fasciatus* BRM-055675, therefore corroborating the works of Laureno-Perez *et al.*[34] and Fillat *et al.*[35]

These results compare with those obtained by Arias *et al.*,[17] who evaluated the performance of enzymatic extracts with surfactants in the hydrolysis of sugarcane bagasse. This previous work achieved a synergism of 1.43 for glucose release by adding PEG 4000 to the *Trichoderma harzianum* extract. Zhang *et al.*[36] observed a synergism of 1.80 when mixing cellulases (from *Thermoascus aurantiacus*) and pectinases (Pectinex Ultra SP-L) for the hydrolysis of hemp pretreated by steam explosion. Hu *et al.*[37] investigated synergism in a mixture of xylanases (Multifect Xylanase, Genencor US Inc., Palo Alto, CA, USA) and cellulases (Celluclast 1.5 L, Novozymes, Franklington, NC, USA) in the hydrolysis of corn straw pretreated by steam explosion and achieved a synergism of 1.62. Pavón-Orozco *et al.*[38] studied synergism between genetically modified strains, *Cellulomonas flavigena* xylanase CflXyn11A and *T. reesei* endoglucanase TrCel7B, for the hydrolysis of pretreated sugarcane bagasse, and achieved a synergism of 6.3.

Enzymatic hydrolysis of BEB

Bunches biologically pretreated with eight macro-basidiomycetes were subjected to enzymatic hydrolysis using Cellic® CTec3® to verify the effect of these extractives on glucose release. The results for the enzymatic saccharification of BEB (with extractives), using the Cellic® CTec3® enzyme (15 FPU g^{-1} dry weight), are shown in Fig. 3. The sugar release was highly dependent on the strains used in BEB. Maximum yields of glucose occurred for *Pleurotus* sp. BRM-060012 of 15.52 ± 0.70% (2.73 ± 0.12 g L^{-1}) and for *F. flavus* of BRM-055676 of 15.03 ± 0.67% (2.76 ± 0.12 g L^{-1}) (Fig. 3(A)). These results indicate that extractive removal increases the glucose release by 36.7% (from 15.0 to 20.5%; Fig. 3(A and C)). Concerning xylose, the highest yield of 15.76 ± 0.39% (1.40 ± 0.03 g L^{-1}) occurred for *Trametes* sp. BRM-060007 (Fig. 3(B)).

These results indicate that the digestibility of the palm oil bunches increased after the biological pretreatment by the fungi *F. flavus* BRM-055676 and *Pleurotus* sp. BRM-060012 (glucose) and *F. flavus* BRM-055676, *Trametes* sp. BRM-060007 and *Pycnoporus* sp. BRM-062381 (xylose). Fungal pretreatment decreases lignin content, modifying the structure of the plant cell wall and resulting in more amorphous regions – instead of the crystalline regions present in the control biomass (untreated), thus improving the accessibility of cellulases and hemicellulases to hollocellulose.[35]

These results also suggest that the de-lignification of the bunches, provided by *F. flavus* BRM-055676 (SLC = 2.96) for 15 days at the established conditions, may have facilitated its digestibility during hydrolysis. Previous studies on enzymatic activities showed that the production of peroxidases by *F. flavus* BRM-055676 could justify its performance on EFB lignin (Silva *et al.*, unpublished results).

An alternative for reducing lignin content in plant biomass could be a pretreatment using ligninolytic enzymes.[39] The addition of mediators 1-hydroxy benzotriazole and manganese sulfate ($MnSO_4$) in the enzymatic extracts produced by *P. sanguineus* UPM4 has been shown to lead to an increase in the activity of ligninolytic enzymes. Then, the palm oil bunches were pretreated with these extracts and then hydrolyzed, leading to significant yields of fermentable sugars.[40] In addition, in optimization studies, improvements in the saccharification of these bunches were due to the previous pretreatment using the laccase enzyme of *Myceliophthora thermophila* 51 003 (Novozymes), among other factors.[41]

Some researchers have focused on the use of microbial consortia, combining the cultivation of white and brown rot fungi, which comprise a viable and promising alternative to improve enzymatic digestibility, reducing the need for long cultivation periods, as usually occurs in conventional biological pretreatments.[42] It is crucial to use fungal strains that could promote the reduction of lignin in a short period. That corroborates Polprasert *et al.*,[11] who evaluated pretreatment of palm EFBs using sequential enzymatic hydrolysis and yeast fermentation, and concluded that the hydrolytic enzyme mixture used enhanced sugar formation compared with a single enzyme.

Enzymatic hydrolysis of BEB pretreated by autohydrolysis

Figure 4(A–H) shows the results of hydrolysis performed with EFBs without pretreatment and subjected to combinations of pretreatments. The biological pretreatment using *F. flavus* BRM-055676 improved the digestibility of EFBs for the CEs, achieving 1.25 ± 0.08 g L^{-1} of glucose, which corresponds to a 6.8% yield increase (Fig. 4(C)). In this case, despite the lower yields, the enzymatic combinations *F. fasciatus* BRM-

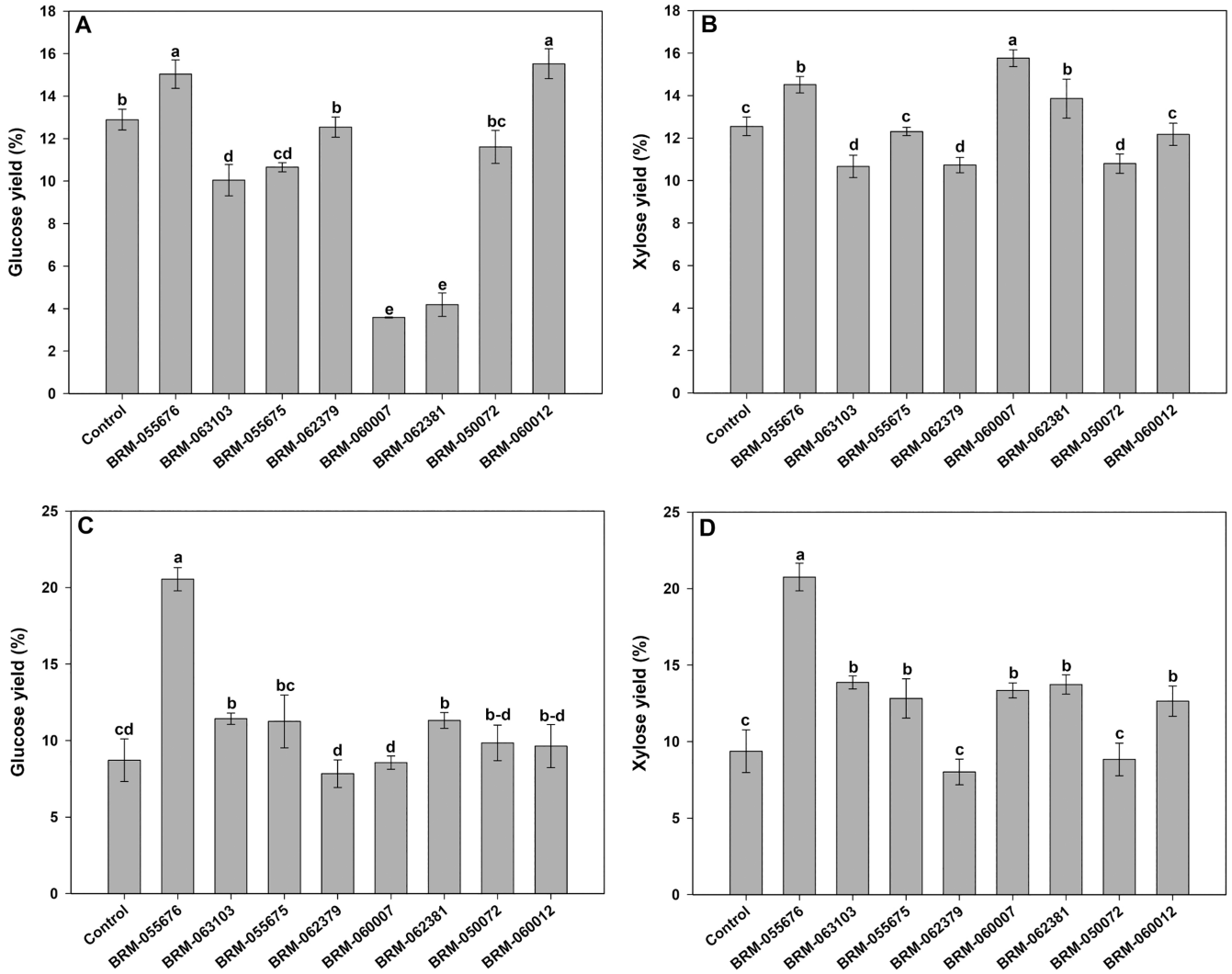


Figure 3. Sugar yield (glucose and xylose) after saccharification of BEB with extractives (A, B) and BEB free of extractives (C, D) after enzymatic hydrolysis at pH 5.0 and 50 °C for 24 h using the Cellic® CTec3® enzyme (Novozymes) (15 FPU g^{-1} dry weight). Yields were calculated based on the levels of glucan and xylan in the BEB. Data shown represent the average and deviations of analyses performed in quadruplicate from each essay performed in triplicate. Different letters indicate statistically significant differences using the Tukey test ($P < 0.05$).

055675 + (Tr + Aa) and *F. flavus* BRM-055676 + (Tr + Aa) led to higher sugar releases when compared with the control (Tr + Aa).

The hydrothermal pretreatment resulted in the highest sugar yields, mainly when using the CE Cellic® CTec3® (Fig. 4(E and F)) compared with the other treatments. It reached 4.45 ± 0.10 g L^{-1} of glucose, which corresponds to a 23.07% yield increase. The mixtures of *F. fasciatus* BRM-055675+ (Tr + Aa) and (Tr + Aa) in combination led to the highest glucose yields of 10.34% (1.99 ± 0.17 g L^{-1}), surpassing the yield of *F. flavus* BRM-055676 + (Tr + Aa) (1.45 ± 0.03 g L^{-1}). Regarding the combined pretreatment (biological + hydrothermal), in general, the glucose yields were close to those achieved with EFB-AH (Fig. 4(E–H)). In addition, comparing the results of raw and pretreated EFBs, the glucose release increased 2.7 times applying hydrothermal pretreatment [from 6.19% to 23.08% with Celic – Fig. 4(A and E)] and 2.5 times with biological + hydrothermal pretreatment [6.19–21.75% with Celic – Fig. 4(A and G)].

The efficiency of macro-basidiomycetes in deconstructing the lignocellulosic biomass has encouraged their use in combination and other pretreatments. The sequential pretreatment (steam + fermentation with *I. lacteus*) of beech wood (*Fagus sylvatica*) achieved higher glucose yields.[43] The combination of physical (particle reduction) and biological pretreatment with the fungus *P. ostreatus* led to a higher methane yield than untreated biomass.[44] However,

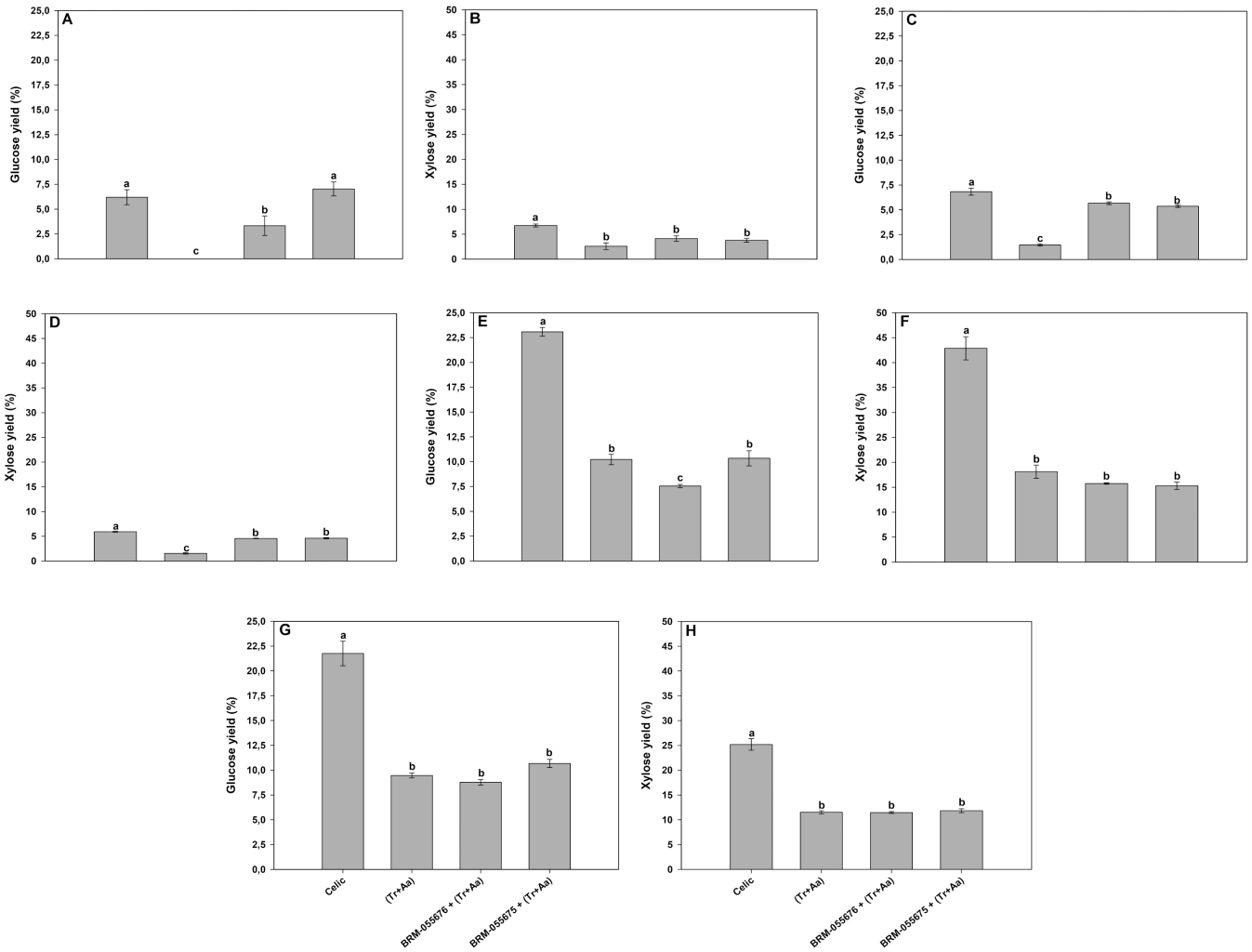


Figure 4. Sugar yield (glucose and xylose) after enzymatic hydrolysis at pH 5.0 and 50 °C for 24 h, comparing Cellic® CTec3® (Novozymes) (15 FPU g^{-1} dry weight) with CEEs of ascomycetes (Tr + Aa) (12.5 mg g^{-1} dry weight) or combinations of CEEs from basidiomycetes [*Flavodon flavus* BRM-055676 (12.69 mL g^{-1} dry weight) + (Tr + Aa) (12.5 mg g^{-1} dry weight) or *Fomes fasciatus* BRM-055675 (12.69 mL g^{-1} dry weight) + (Tr + Aa) (12.5 mg g^{-1} dry weight)], on pretreated and untreated empty bunches. (A, B) Raw empty fruit bunches (EFBs); (C, D) treated with *F. flavus* BRM-055676 (BEB); (E, F) bunches pretreated by autohydrolysis (EFB-AH); and (G, H) bunches pretreated by *F. flavus* BRM-055676 and autohydrolysis. Data shown represent the average deviations of analyses performed in triplicate from each essay also performed in triplicate. Different letters indicate statistically significant differences using the Tukey test ($P < 0.05$).

an effective biological pretreatment does not require a reduction in the biomass particles to guarantee the pentose (hemicellulose) fractions, avoid the formation of microbial inhibitors and reduce costs. The breakdown and removal of lignin from biomass is the goal for using basidiomycetes. This polymer contributes to the recalcitrance of the material, hindering enzymatic action during the saccharification stage of polysaccharides (cellulases).[45]

Among the factors that limit hydrolysis are cellulose crystallinity, degree of polymerization and lignin content on the surface.[34] To overcome these issues, the use of biological pretreatments with white-rot fungi represents a low-cost and environmentally friendly alternative.[46] With a direct effect on the recovery of sugars to obtain ethanol, bio-delignification was used before chemical pretreatments.[47] The combination of pretreatments is a strategy that aims at increasing the yield of saccharification of lignocellulosic biomasses.[48] However, it is still necessary to optimize the cultivation conditions to reduce the time of colonization of the substrate and provide the highest sugar yields.

Reduction of enzymatic hydrolysis inhibitors

During hydrothermal pretreatment, the production of several soluble inhibitors affects the enzymatic hydrolysis and fermentation efficiency, such as phenolic inhibitors derived from lignin.[4] The results on the potential reduction of phenolic substances by enzymatic action obtained in this present study are provided in Table 4. The percentages of reduction of compounds depend on the treatment. CEEs containing only enzymes from *T. reesei* and *A. aculeatus* reduced levels of all substances, mainly coumaric acid (79.9%). In some cases, combination with CEEs of macro-basidiomycetes provided large reductions in some of them. The addition of *F. fasciatus* BRM-055675 CEEs significantly reduced the concentrations of gallic and synaptic acid.

Regarding gallic acid, the combination *F. fasciatus* BRM-055675 + (Tr + Aa) led to a reduction of 56.5%, while (Tr + Aa) provided a reduction of 51.7%. The CEEs of *F. fasciatus* also reduced the concentration of synaptic acid, reducing it to 71.4%, compared with a reduction of 56.7% provided by the combination *F. flavus* BRM-055676 + (Tr + Aa) and 51.8% by (Tr + Aa). Regarding succinic acid, the combination *F. flavus* + (Tr + Aa) reduced it the most (46.7%), followed by *F. fasciatus* + (Tr + Aa) (45.02%) and (Tr + Aa) (43.6%). The CEEs of macro-basidiomycetes alone promoted lower reductions at the evaluated conditions when compared with the (Tr + Aa).

Phenolic compounds generated during the pretreatment of lignocellulosic biomass reduce the activity of cellulases and can also inhibit bacteria and yeasts in fermentation processes.[49] Phenols, such as vanillin, syringaldehyde, *trans*-cinnamic acid and hydroxybenzoic acid, for example, inhibit cellulose hydrolysis in wet corn cake.[50] These substances can alter the structural conformation of proteins, leading to their inactivation.[51] According to Toquero and Bolado,[52] yeasts may suffer stress because of high osmotic pressure or high concentrations of inhibitory compounds, and the combination of these factors can act synergistically, affecting ethanol yields. Although some studies indicate the causes of the decrease in hydrolysis efficiency, little is known about the mechanisms involved in the enzymatic inhibition process.[53]

Inhibitors such as furaldehydes, phenolic compounds and organic acids were released in biomass pretreatment and hydrolysis during the deconstruction of cellulose and hemicellulose into monomeric sugars. These inhibitors unsettle microbial metabolism by damaging cellular membranes and reducing intracellular pH, affecting enzymes activities, which may result in a prolonged lag phase, lower fermentation rates and reduced tolerance to ethanol.[54] Macro-basidiomycetes have developed biochemical abilities to deal with the substances resulting from lignin degradation and plant defenses.[55] Part of the success in dealing with toxic substances has been associated with cytochrome P450 monooxygenases, constituting the fungal defense system against xenobiotics.[56]

Table 4. Reduction (%) of phenolic and inhibitory substances by CEEs of basidiomycetes and ascomycetes.

Phenolic compounds	(Tr + Aa)	*Flavodon flavus* BRM-055676	(Tr + Aa) + *F. flavus* BRM-055676	*Fomes fasciatus* BRM-055675	(Tr + Aa) + *F. fasciatus* BRM-055675
Succinic acid	43.65 ± 0.19^{c}	4.22 ± 0.33^{e}	46.71 ± 0.38^{a}	6.94 ± 0.60^{d}	45.02 ± 0.39^{b}
Lactic acid	43.42 ± 0.48^{a}	0.00 ± 0.00^{b}	41.06 ± 0.53^{a}	1.52 ± 1.18^{b}	42.01 ± 0.84^{a}
Formic acid	43.33 ± 0.73^{a}	0.00 ± 0.00^{d}	38.52 ± 0.65^{c}	0.00 ± 0.00^{d}	40.05 ± 0.13^{b}
Acetic acid	42.97 ± 0.80^{a}	0.00 ± 0.00^{c}	40.08 ± 0.35^{b}	0.29 ± 0.41^{c}	42.54 ± 0.15^{a}
Levulinic acid	40.53 ± 1.31^{a}	1.33 ± 0.44^{b}	38.47 ± 0.63^{a}	2.33 ± 0.53^{b}	40.53 ± 0.83^{a}
Galic acid	51.77 ± 1.48^{b}	8.70 ± 0.68^{d}	46.97 ± 1.54^{c}	2.63 ± 1.46^{e}	56.55 ± 0.52^{a}
HMF	49.28 ± 0.81^{a}	7.66 ± 0.05^{b}	47.95 ± 1.19^{a}	8.78 ± 1.75^{b}	50.55 ± 0.61^{a}
Furfural	48.16 ± 0.35^{a}	1.35 ± 1.11^{b}	47.28 ± 0.50^{a}	1.76 ± 0.91^{b}	47.58 ± 0.72^{a}
Vanillic acid	50.10 ± 1.31^{a}	9.02 ± 033^{b}	48.07 ± 1.17^{a}	0.00 ± 0.00^{c}	50.67 ± 0.49^{a}
Vanillin	49.10 ± 1.07^{a}	4.44 ± 0.19^{d}	46.11 ± 0.89^{b}	8.57 ± 0.98^{c}	49.36 ± 0.14^{a}
Coumaric acid	79.97 ± 0.41^{a}	7.01 ± 0.17^{c}	48.30 ± 0.89^{b}	0.00 ± 0.00^{d}	49.74 ± 0.99^{b}
Syringaldehyde	48.30 ± 0.93^{a}	5.43 ± 0.21^{b}	46.80 ± 1.08^{a}	0.00 ± 0.00^{c}	49.03 ± 0.57^{a}
Ferulic acid	49.38 ± 1.32^{a}	8.12 ± 0.01^{b}	48.51 ± 0.97^{a}	0.00 ± 0.00^{c}	50.91 ± 0.42^{a}
Synaptic acid	51.85 ± 1.41^{b}	15.64 ± 0.14^{d}	56.72 ± 1.19^{b}	28.05 ± 2.73^{c}	71.46 ± 0.24^{a}

Inhibitor quantification of enzymatic hydrolysis performed at pH 5.0 and 50 °C for 180 min, using a combination of CEEs of basidiomycete and ascomycetes. Data shown represent the averages and deviations of analyzes performed in triplicate from each essay also performed in triplicate. Different letters in the column indicate statistically significant differences using the Tukey test ($P < 0.05$).

There is a reduction in some phenolic compounds when using the CEEs of macrobasidiomycetes and ascomycetes (Fig. 4). The basidiomycete *F. fasciatus* BRM-055675 proved to be a good option for further studies regarding inhibitors. It is still necessary to optimize its use and build a 72 h analytical curve of degradation to evaluate enzymatic hydrolysis of previously pretreated plant biomass.

Conclusions

This study presents an evaluation of the deconstruction of oil palm EFBs. The autohydrolysis pretreatment showed the best sugar yields during hydrolyses; however, biological pretreatment provides enzymes and other advantages. The biological pretreatment of EFBs by *F. flavus* showed higher selectivity over the lignin. The higher synergism degree for glucose release from EFB-AH resulted from the enzymatic extract of *F. fasciatus* combined with CEs. The cocktail of *F. flavus* and commercial enzymes reached the highest glucose yield during hydrolyses of EFB-AH. The *F. fasciatus* enzymatic extract demonstrated potential for phenolic substance reduction. Therefore, based on these results, we can postulate that the use of basidiomycetes from the Brazilian cerrado to pretreat empty bunches of palm oil has a high biotechnological potential for biorefinery purposes.

Acknowledgement

The authors acknowledge funding to E.A.S. from the Coordination for the Improvement of Higher Education Personnel, a Foundation within the Ministry of Education in Brazil, via the Graduate Program in Plant Biotechnology, Federal University of Lavras. The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: the grant (01.13.0315.00 DendePalm Project) for this study was awarded by the Brazilian Ministry of Science, Technology and Innovation (MCTI) via the Brazilian Innovation Agency FINEP. The authors confirm that the funder had no influence over the study design, the content of the article, or selection of this journal.

Conflict of interest

The authors declare that they have no conflicts of interest.

Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors. The authors confirm that principles of ethical and professional conduct have been followed in this research and in the preparation of this article.

References

1. Van Dyk JS and Pletschke BI, A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes – Factors affecting enzymes, conversion and synergy. *Biotechnol Adv* **30**(6):1458–1480 (2012).
2. Abdul Khalil HPS, Alwani M, Ramli R, Kamarudin H and Khairul A, Chemical composition, morphological characteristics, and cell wall structure of Malaysian oil palm fibers. *Polym Plast Technol Eng* **47**:273–280 (2008).
3. Amelia CKM, Ng LY, Ng CY, Mahmoudi E, Hairom NHH and Mah SK, Polyethersulfone-cellulose composite thin film incorporated with regenerated-cellulose extracted from empty fruit bunches of *Elaeis guineensis*. *Mater Today Proc* **46**:1882–1888 (2021).
4. Akhlisah ZN, Yunus R, Abidin ZZ, Lim BY and Kania D, Pretreatment methods for an effective conversion of oil palm biomass into sugars and high-value chemicals. *Biomass Bioenergy* **144**(105):901 (2021).
5. Sindhu R, Binod P and Pandey A, Biological pretreatment of lignocellulosic biomass – An overview. *Bioresour Technol* **199**:76–82 (2016).
6. Shirkavand E, Baroutian S, Gapes DJ and Young BR, Combination of fungal and physicochemical processes for lignocellulosic biomass pretreatment – A review. *Renew Sustain Energy Rev* **54**:217–234 (2016).
7. Saha BC, Qureshi N, Kennedy GJ and Cotta MA, Biological pretreatment of corn stover with white-rot fungus for improved enzymatic hydrolysis. *Int Biodeterior Biodegradation* **109**:29–35 (2016).
8. Mamimin C, Chanthong S, Leamdum C, Sompong O and Prasertsan P, Improvement of empty palm fruit bunches biodegradability and biogas production by integrating the straw mushroom cultivation as a pretreatment in the solid-state anaerobic digestion. *Bioresour Technol* **319**(124):227 (2021).
9. García-Torreiro M, López-Abelairas M, Lu-Chau TA and Lema JM, Fungal pretreatment of agricultural residues for bioethanol production. *Ind Crops Prod* **89**:486–492 (2016).
10. Saha BC, Kennedy GJ, Qureshi N and Cotta MA, Biological pretreatment of corn stover with *Phlebia brevispora* NRRL-13108 for enhanced enzymatic hydrolysis and efficient ethanol production. *Biotechnol Prog* **33**(2):365–374 (2017).
11. Polprasert S, Choopakar O and Elefsiniotis P, Bioethanol production from pretreated palm empty fruit bunch (PEFB) using sequential enzymatic hydrolysis and yeast fermentation. *Biomass Bioenergy* **149**(106):088 (2021).
12. Wang Y, Shao Y, Zou X, Yang M and Guo L, Synergistic action between extracellular products from white-rot fungus and cellulase significantly improves enzymatic hydrolysis. *Bioengineered* **9**(1):178–185 (2018).
13. Mandel M and Weber J, The production of cellulases, in *Cellulases and its Application. Advances in Chemistry Series*. American Chemical Society, Washington, DC, pp. 391–414 (1969).
14. Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J and Templeton D, *Determination of Ash in Biomass: Laboratory Analytical Procedure (LAP)*. National Renewable Energy Laboratory, Golden, CO, (2008a).
15. Sluiter A, Ruiz R, Scarlata C, Sluiter J and Templeton D, *Determination of Extractives in Biomass*. Laboratory Analytical Procedure (LAP). National Renewable Energy Laboratory, Golden, CO, (2008b).

16. Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J, Templeton, D. and Crocker, D. (2012) NREL/TP-510-42618 analytical procedure – Determination of structural carbohydrates and lignin in biomass. Laboratorial Analysis Procedure, 17.
17. Arias JM, De Oliveira Moraes A, Modesto LFA, De Castro AM and Pereira N Jr, Addition of surfactants and non-hydrolytic proteins and their influence on enzymatic hydrolysis of pretreated sugarcane bagasse. *Appl Biochem Biotechnol* **181**(2):593–603 (2017).
18. Xiao Z, Storms R and Tsang A, Microplate-based filter paper assay to measure total cellulase activity. *Biotechnol Bioeng* **88**:832–837 (2004).
19. Smith PE, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano M *et al*., Measurement of protein using bicinchoninic acid. *Anal Biochem* **150**(1):76–85 (1985).
20. Ying TY, Teong LK, Abdullah WNW and Peng LC, The effect of various pretreatment methods on oil palm empty fruit bunch (EFB) and kenaf core fibers for sugar production. *Procedia Environ Sci* **20**:328–335 (2014).
21. Thamvithayakorn P, Phosri C, Pisutpaisal N, Krajangsang S, Whalley AJ and Suwannasai N, Utilization of oil palm decanter cake for valuable laccase and manganese peroxidase enzyme production from a novel white-rot fungus, *Pseudolagarobasidium* sp. PP17-33. *3 Biotech* **9**:417 (2019).
22. Jung YR, Park JM, Heo SY, Hong WK, Lee SM, Oh BR *et al*., Cellulolytic enzymes produced by a newly isolated soil fungus *Penicillium* sp. TG2 with potential for use in cellulosic ethanol production. *Renew Energy* **76**:66–71 (2015).
23. Isroi I, Millati R, Niklasson C, Cayanto C, Taherzadeh MJ and Lundquist K, Biological treatment of lignocelluloses with white-rot fungi and its applications. *BioResources* **6**(4):5224–5259 (2011).
24. Kamcharoen A, Champreda V, Eurwilaichitr L and Boonsawang P, Screening and optimization of parameters affecting fungal pretreatment of oil palm empty fruit bunch (EFB) by experimental design. *International Journal of Energy and Environmental Engineering* **5**(4):303–312 (2014).
25. Ghasemzadeh R, Mosavian MTH and Karimi A, Analysis of biological pretreatment of rapeseed straw with white-rot fungi for enzymatic hydrolysis. *Maderas Cienc Tecnol* **20**(4):725–736 (2018).
26. Wan C and Li Y, Fungal pretreatment of lignocellulosic biomass. *Biotechnol Adv* **30**(6):1447–1457 (2012).
27. De La Torre I, Ravelo M, Segarra S, Tortajada M, Santos VE and Ladero M, Study on the effects of several operational variables on the enzymatic batch saccharification of orange solid waste. *Bioresour Technol* **245**:906–915 (2017).
28. Ramos LP, Da Silva L, Ballem AC, Pitarelo AP, Chiarello LM and Silveira MHL, Enzymatic hydrolysis of steam-exploded sugarcane bagasse using high total solids and low enzyme loadings. *Bioresour Technol* **175**:195–202 (2015).
29. Eibinger M, Bubner P, Ganner T, Plank H and Nidetzky B, Surface structural dynamics of enzymatic cellulose degradation, revealed by combined kinetic and atomic force microscopy studies. *FEFBS J* **281**(1):275–290 (2014).
30. Wang H, Kobayashi S and Mochidzuki K, Effect of non-enzymatic proteins on enzymatic hydrolysis and simultaneous saccharification and fermentation of different lignocellulosic materials. *Bioresour Technol* **190**:373–380 (2015).
31. Kun RS, Gomes ACS, Hildén KS, Cerezo SS, Mäkelä MR and De Vries RP, Developments and opportunities in fungal strain engineering for the production of novel enzymes and enzyme cocktails for plant biomass degradation. *Biotechnol Adv* **37**(6):107361 (2019).
32. Skyba O, Cullen D, Douglas CJ and Mansfield SD, Gene expression patterns of wood decay fungi *Postia placenta* and *Phanerochaete chrysosporium* are influenced by wood substrate composition during degradation. *Appl Environ Microbiol* **82**(14):4387–4400 (2016).
33. Kumar R and Wyman CE, Effect of additives on the digestibility of corn stover solids following pretreatment by leading technologies. *Biotechnol Bioeng* **102**(6):1544–1557 (2009).
34. Laureno-Perez L, Teymouri F, Alizadeh H and Dale B, Understanding factors that limit enzymatic hydrolysis of biomass characterization of pretreated corn stover. *Appl Biochem Biotechnol* **124**:1081–1099 (2005).
35. Fillat Ú, Ibarra D, Eugenio ME, Moreno AD, Tomás-Pejó E and Martín-Sampedro R, Laccases as a potential tool for the efficient conversion of lignocellulosic biomass: A review. *Fermentation* **3**(2):17 (2017).
36. Zhang J, Pakarinen A and Viikari L, Synergy between cellulases and pectinases in the hydrolysis of hemp. *Bioresour Technol* **129**:302–307 (2013).
37. Hu J, Arantes V and Saddler JN, The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect?. *Biotechnol Biofuels* **4**:36 (2011).
38. Pavón-Orozco P, Santiago-Hernández A, Rosengren A, Hidalgo-Lara ME and Stålbrand H, The family II carbohydrate-binding module of xylanase CflXyn11A from *Cellulomonas flavigena* increases the synergy with cellulase TrCel7B from *Trichoderma reesei* during the hydrolysis of sugar cane bagasse. *Bioresour Technol* **104**:622–630 (2012).
39. Giacobbe S, Pezzella C, Lettera V, Sannia G and Piscitelli A, Laccase pretreatment for agrofood wastes valorization. *Bioresour Technol* **265**:59–65 (2018).
40. Zanirun Z, Bahrin EK, Lai-Yee P, Hassan MA and Abd-Aziz S, Enhancement of fermentable sugars production from oil palm empty fruit bunch by ligninolytic enzymes mediator system. *Int Biodeterior Biodegradation* **105**:13–20 (2015).
41. Ishmael UC, Shah RS, Palliah JV, Asras MFF, Ahmad SS and Ayodele VB, Statistical modeling and optimization of enzymatic pretreatment of empty fruit bunches with laccase enzyme. *BioResources* **11**(2):5013–5032 (2016).
42. Kalyani D, Lee KM, Kim TS, Li J, Dhiman SS, Kang YC *et al*., Microbial consortia for saccharification of woody biomass and ethanol fermentation. *Fuel* **107**:815–822 (2013).
43. Brethauer S, Lawrence SR and Hans-Peter SM, Enhanced simultaneous saccharification and fermentation of pretreated beech wood by in situ treatment with the white-rot fungus *Irpex lacteus* in a membrane aerated biofilm reactor. *Bioresour Technol* **237**:135–138 (2017).
44. Mustafa AM, Poulsen TG, Xia Y and Sheng K, Combinations of fungal and milling pretreatments for enhancing rice straw biogas production during solid-state anaerobic digestion. *Bioresour Technol* **224**:174–182 (2017).
45. Wagner AO, Lackner N, Mutschlechner M, Prem EM, Markt R and Illmer P, Biological pretreatment strategies for second-generation lignocellulosic resources to enhance biogas production. *Energies* **11**(7):1797 (2018).
46. Akhtar J and Idris A, Oil palm empty fruit bunches a promising substrate for succinic acid production via simultaneous saccharification and fermentation. *Renew Energy* **114**:917–923 (2017).
47. Yu H, Du W, Zhang J, Ma F, Zhang X and Zhong W, Fungal treatment of cornstalks enhances the delignification and xylan loss during mild alkaline pretreatment and enzymatic digestibility of glucan. *Bioresour Technol* **101**(17):6728–6734 (2010).

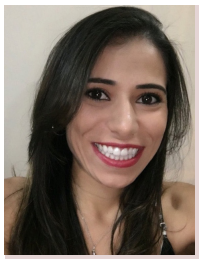
48. Hsu T-A, Pretreatment of biomass, in *Handbook on Bioethanol*. Routledge, Boca Raton, FL, pp. 179–212 (2018).
49. Rasmussen H, Tanner D, Sørensen HR and Meyer AS, New degradation compounds from lignocellulosic biomass pretreatment: routes for formation of potent oligophenolic enzyme inhibitors. *Green Chem* **19**(2):464–473 (2017).
50. Ximenes E, Kim Y, Mosier N, Dien B and Ladisch M, Inhibition of cellulases by phenols. *Enzyme Microb Technol* **46**(3–4):170–176 (2010).
51. Boukari I, O'donohue M, Rémond C and Chabbert B, Probing a family GH11 endo-β-1, 4-xylanase inhibition mechanism by phenolic compounds: role of functional phenolic groups. *J Mol Catal B Enzym* **72**(3–4):130–138 (2011).
52. Toquero C and Bolado S, Effect of four pretreatments on enzymatic hydrolysis and ethanol fermentation of wheat straw. Influence of inhibitors and washing. *Bioresour Technol* **157**:68–76 (2014).
53. Ázar RIL, Morgan T, Dos Santos ACF, De Aquino Ximenes E, Ladisch MR and Guimarães VM, Deactivation and activation of lignocellulose degrading enzymes in the presence of laccase. *Enzyme Microb Technol* **109**:25–30 (2018).
54. Pacheco TF, Machado BRC, De Morais Júnior WG, Almeida JR and Gonçalves SB, Enhanced tolerance of *Spathaspora passalidarum* to sugarcane bagasse hydrolysate for ethanol production from xylose. *Appl Biochem Biotechnol* **193**:2182–2197 (2021).
55. Schmidt-Dannert C, Biocatalytic portfolio of Basidiomycota. *Curr Opin Chem Biol* **31**:40–49 (2016).
56. Ichinose H, Cytochrome P 450 of wood-rotting basidiomycetes and biotechnological applications. *Biotechnol Appl Biochem* **60**(1):71–81 (2013).

Elias Alves da Silva

Elias Alves da Silva graduated in Biological Sciences from the University Center of Formiga, Minas Gerais (UNIFOR-MG 2010), and is a Specialist in Science Teaching by Investigation at the Federal University of Minas Gerias (UFMG 2014). He gained a Master's in Medicinal, Aromatic and Seasoning Plants at the Federal University of Lavras (UFLA, 2015) and a PhD in Plant Biotechnology at the Federal University of Lavras in partnership with the Brazilian Agricultural Research Corporation – Agroenergy Unit (2019).

Thais Demarchi Mendes

Thais Demarchi Mendes graduated in Biology (UNESP/Brazil – 2008) and gained a Master's in Applied Microbiology from the same university (2010). Since 2011 she has been working as a Research and Development Analyst at Embrapa Agroenergy. She works in the field of applied microbiology, development of fermentation processes and enzymatic technology.



Thályta Fraga Pacheco

Thályta Fraga Pacheco graduated in Chemical Engineering and gained a Master's degree in Biochemical Process Development, and has been working since 2010 as a Research and Development Analyst at Embrapa Agroenergy on the development and optimization of fermentation processes for different purposes.

Raquel Bombarda Campanha

Raquel Bombarda Campanha obtained her Bachelor's Degree in Chemistry from the University of São Paulo (2005) and a Master's Degree in Food Science and Engineering from São Paulo State University (2010). She is currently working as research analyst at the Brazilian Agricultural Research Corporation (Embrapa). She has experience in chemistry, with emphasis on plant biomass chemistry and analytical and instrumental chemistry.

Daiana Wischral

Daiana Wischral graduated in Food Engineering and has a PhD in Chemical and Biochemical Process Technology from the Federal University of Rio de Janeiro (Brazil) in partnership with the Ohio State University, Columbus, Ohio (USA). She is a consultant at Embrapa Agroenergy (Brazil), working with microbial fermentation of enzymes and secondary metabolite production.

Simone Mendonça

Simone Mendonça graduated in Pharmacy–Biochemistry, with a Master's in Food Science and Technology (Gent, Belgium) and a PhD in Nutrition (Sao Paulo University, Brazil). She is a researcher at Embrapa Agroenergia (Brazil) and a specialist in biomass chemistry, bioactive compounds and biorefinery approaches in valorization of co-products of the biofuels chain.

Marli Camassola

Marli Camassola is a professor at the University of Caxias do Sul, Brazil. She received her PhD degree in Biotechnology in 2007. She works with fungal biotechnology intended for various industrial sectors such as food, pharmaceuticals, cosmetics and bioremediation, using agro-industrial by-products for developing new sustainable products and processes.

Félix Gonçalves de Siqueira

Félix Gonçalves de Siqueira graduated in Biology, and received a Master's in Agricultural Microbiology (UFLA-Brazil) and a PhD in Molecular Biology and Enzymology (UNB-Brazil) as part of work done at Purdue University (USA). He is currently a researcher at Embrapa Agroenergia (Brazil), in the area of microbial fermentation (enzymes and secondary metabolites) and biorefinery approaches.

Manoel Teixeira Souza Júnior

Manoel Teixeira Souza Júnior is a researcher at Embrapa Agroenergy and a Professor in the Graduate Program on Plant Biotechnology at the Federal University of Lavras. His research is related to the use of biotechnology to improve the production and utilization of oil palm biomass.