Received: 28 May 2021 | Revised: 20 January 2022 | Accepted: 25 January 2022

DOI: 10.1002/ese3.1093

Energy Science & Engineering Open Access

ORIGINAL ARTICLE

Colonization of oil palm empty fruit bunches by basidiomycetes from the Brazilian cerrado: Enzyme production

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Funding information
The author(s) 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

Abstract

The use of residual lignocellulosic biomass appears as an opportunity to obtain high added value products like enzymes. The present work goal was to select basidiomycetes capable of metabolizing empty fruit bunches (EB) or sludge decanter (SD) from the oil palm industry to produce enzymatic cocktails. First, eight macro-basidiomycetes strains from the Brazilian cerrado were selected based on genomic DNA analyses out of an initial group of 30. The selected basidiomycetes and two ascomycetes (commercial mushrooms) were cultured to obtain ligninolytic and cellulolytic enzymes, respectively. All the extracts produced had proteins, and enzymatic profiles evaluated. The enzymatic hydrolysis of pretreated EB used cocktails of basidiomycetes extracts combined with commercial cellulases or ascomycetes extracts. The best combination of enzymatic extracts for hydrolyses of hydrothermally pretreated EB was the cocktail with *Trichoderma reesei*, *Aspergillus aculeatus*, and *Pleurotus* sp. extracts, reaching 19.7% of glucose yield and 22.8% of xylose yield. These results show that enzymes and sugars are products derivable from residues produced in large quantities by the oil palm industry. This study corroborates the hypothesis that basidiomycetes from the Brazilian cerrado present an enormous biotechnological potential, still unexplored.

KEYWORDS

basidiomycetes, biological pretreatment, biorefinery, delignification, *Elaeis* spp, enzymatic hydrolysis

1 | INTRODUCTION

Energy production from renewable sources is a subject that has grown in importance worldwide, mainly among the most developed countries.[1] The focus is on developing a sustainable bioeconomy using natural resources consciously and preserving the environment. Within this scenario, under the scope of biorefineries, lignocellulosic biomass plays an important role. It is the raw material for converting cellulose and hemicellulose (polysaccharides) into fermentable sugars, as well as value-added chemicals.[2] However, lignin increases the recalcitrance of this biomass, making enzymatic hydrolyses more difficult.[3] Severe pretreatments—acid, alkaline, and high-temperature steam—are the way to overcome that recalcitrance. The goal is to deconstruct the cell wall, allowing the rupture of polysaccharide chains through enzymatic hydrolysis.[2]

Nevertheless, some bottlenecks remain while looking forward to obtaining soluble sugars from the combination of pretreatment and enzymatic hydrolysis techniques. Biological pretreatment using white rot fungi or combining it with chemical and physical pretreatments can be alternatives to increase the yield of hexose and pentose from holocellulose.[4] Basidiomycetes present an enzyme apparatus rich in lignocellulolytic enzymes, making them the significant natural decomposition agents of cell wall plants.[5] Using these fungi for pretreatment of plant biomass presents the advantage of generating fewer effluents than chemical washing and lowering costs.[6] However, the longer time needed for colonization of these fungi remains a disadvantage of this process.[5]

White rot fungi have been extensively studied in recent years, searching for species with a specific lignin/cellulose (SLC) selectivity pattern during lignocellulosic deconstruction.[5] Accessory enzyme-producing genes, as lytic polysaccharide monooxygenases (LPMOs) and expansins, are part of the genome of most basidiomycetes, leading to the production of an enzyme repertoire depending on the substrate characteristics and cultivation conditions. That has encouraged exploratory studies of the enzyme pool produced during cultivation to evaluate which enzymes are present in the crude extract and how they interact with other enzymes. Thus, some initiatives have suggested mixing these crudes enzyme extracts in hydrolytic combinations or cocktails to investigate possible synergistic actions with cellulases and, consequently, savings in using high concentrations of commercial enzymes.[7]

Residual biomass from agroindustry and woody material, in general, present some differences concerning the structure and chemical composition, the reason why, depending on the material, the behavior of fungal strains may vary.[4] For example, in Thailand, steams and electricity have been produced from palm oil mills bio-waste for decades, enhancing power generation and promoting sustainability.[8] The palm oil industry generates tons of lignocellulosic residues, such as empty fruit bunches (EB) and sludge decanter (SD). The former corresponds to approximately 20% of fresh fruit bunches, and the latter results from the oil clarification step, being deposited at the bottom of the oil decanter, rich in proteins and favors that make this biomass promising for enzymes production.[9]

In 2019, the worldwide production of palm oil was 75.7 million tons,[10] with an estimated 1.5 million tons of EB. According to The Atlas of Economic Complexity,[11] worldwide exportation of palm oil (1511 HS4) has increased 4.7 times between 1995 and 2018, achieving, in 2018, revenues of U$ 29.2 billion. This expansion comprises abundant biomass whose improper disposal could cause environmental damage.[9] Palm oil is the most consumed vegetable oil worldwide, and oil palm has the best income in oil per hectare. Most recently, the biodiesel industry is encouraging sustainable palm oil production, which promotes stability and balance between economic, social, and environmental goals.[12] Brazil imports around 180 thousand tons of palm oil yearly to supply an internal market potential demand of 400 thousand tons/year.[13] Almost all Brazilian palm oil cultivation area is in the state of Pará in the North Region of the country. As part of an initiative to promote the sustainable production and use of this palm in Brazil, Embrapa did the agro-ecological zoning (ZAE) study, revealing an extensive area suitable to the production of oil palm in Brazil.[14]

According to Da Silva Machado and Ferraz,[15] combinations of biological pretreatment and physical or chemical can be interesting strategies to enhance the effectiveness of enzymatic cocktails. This present work aimed at (a) evaluating strains of basidiomycetes from the Brazilian cerrado biodiversity still unexplored, (b) identifying those with high ligninolytic capacity and little holocellulolytic activity when cultivated in empty fruit bunches of oil palm; (c) verifying the amount of sugar released when treating it with enzymatic cocktails resulted from the combination of basidiomycetes with commercial cellulases or ascomycetes extracts.

2 | MATERIALS AND METHODS

2.1 | Strains and plate growth experiments

Thirty basidiomycete strains from the Embrapa Agroenergy Collection, originally isolated from the Brazilian cerrado—or purchased at the local market—were used in this study: BRM-063110, BRM-055676,

BRM-063111, BRM-062374, BRM-062450, BRM-063103, BRM-055675, BRM-062452, BRM-063104, BRM-062453, BRM-062377, BRM-062379, BRM-062380, BRM-060007, BRM-062381, BRM-060008, BRM-063105, BRM-062382, BRM-063106, BRM-060009, BRM-063107, BRM-050072, BRM-060010, BRM-063108, BRM-060011, BRM-063109, BRM-055677, BRM-060012, BRM-062454, and BRM-060013 (http://alelomicro.cenargen.embrapa.br). All strains were stored at 4.0°C in Petri dishes with potato dextrose agar (PDA) and cultured periodically. Inoculum of each fungus was cultivated, from stocks, in Petri dishes containing PDA for 10 days.

DENPASA (Dendê do Pará S/A - www.denpasa.com.br/en-us) provided the empty fruit bunches (EB) and sludge (SD - residual material generated after the oil clarification step) used in this study. These biomasses were dried initially at 45°C for 3 days, later at 60°C for a similar period, and then grounded (80 mesh). Then, part of the biomass was powdered in a Willey Star FT-60 knife mill (≅696 μm) to prepare culture medium on Petri dishes containing agar, and the remainder for cultivation in solid-state, hydrothermal pretreatment, and enzymatic hydrolysis.

Three culture media were prepared with enriched agar for cultivation on plate: (1) EB (powder) 10% w/v and agar 1.5% w/v; (2) EB 10% w/v, SD 10% w/v and agar 1.5% w/v; and (3) PDA (control). The culture media were sterilized at 121°C and 1 atm for 30 min and then transferred to Petri dishes.

2.2 | Selection of basidiomycetes and solid-state cultivation (SC)

First, growth capacity and mycelial vigor were measured when cultivating the basidiomycetes on (a) EB (powder) 10% w/v and agar 1.5% w/v; (b) EB 10% w/v, SD 10% w/v and agar 1.5% w/v; and (c) PDA (control). A 7.0 mm diameter mycelial disk was used as inoculum on each plate and kept in an incubator chamber at 28°C. Mycelial growth was measured every 2 days, using a pachymeter to measure the mycelial growth radius (cm). To calculate mycelial vigor (visual), we used a scale of 0 to 5 points, where 0 indicates no vigor, and 5 indicates excellent vigor. The selected strains—showing greater radius and mycelial vigor on medium enriched with lignocellulosic substrates—were later cultivated in flasks containing biomasses in solid-state cultivation (SC).

The biomasses (EB and SD) were placed in 250 ml Erlenmeyer flasks, comprising two SC culture media: (SC-1) 30 g of EB; (SC-2) 30 g of EB mixed with SD at 2:1 ratio (w/w). Humidity was adjusted to 65%–70% with the addition of distilled water, followed by sterilization for 30 min at 121°C and 1 atm, and then inoculation and cultivation for 30 days at 28°C. Selected strains grew in a modified SC-1 medium (10 g of EB) for 21 days to optimize the process of substrate consumption and to reach maximum enzyme production; while maintaining the other cultivation conditions.

2.3 | Molecular identification of selected basidiomycetes

After crushing the mycelium in a crucible with liquid nitrogen, we used phenol/chloroform[16] to extract the deoxyribonucleic acid (DNA). After treating DNA samples with bovine pancreatic ribonuclease (RNAse A) (Thermo®), we evaluated their quality evaluated on a 1% agarose gel in electrophoresis using DNA from lambda bacteriophage at known marker concentrations.

The ITS1-F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) primers[17] and ITS4-R (5′-TCCTCCGCTTATTGATATG C-3′)[18] were employed to amplify the ITS1-5.8S-ITS2 region, which is approximately 600 bp long. We used a 1% agarose gel stained with ethidium bromide to visualize the amplicons and then photo-documented it under ultraviolet light. These products (100 μl) were purified using Quick Gel Extraction and polymerase chain reaction (PCR) Purification Kit (Invitrogen®). Samples were sequenced at Myleus Biotechnology (www.myleus.com) through capillary electrophoresis using ABI3730 equipment. Polymers used for the sequencing process were POP7 and BigDye v3.1. The obtained sequences were analyzed using the Geneious software (version R11) and compared to the databases: National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov), CBS (www.westerdijkinstitute.nl/collections), and Barcode of Life Data (BOLD) Systems (http://www.boldsystems.org/; making it possible to identify the strains through Basic Local Alignment Search Tool (BLAST).

2.4 | Production of crude enzyme extracts (CEE) by basidiomycetes and ascomycetes

We measured the activities of some lignocellulolytic enzymes (cellulases, xylanases, and ligninases) and the concentration of total soluble proteins from samples obtained from the culture of the following strains at 6, 13, and 21 days of culture: BRM-055676, BRM-063103, BRM-055675, BRM-062379, BRM-060007, BRM-062381, BRM-050072, and BRM-060012, at SC-1(modified) condition. The colonized biomass underwent washing with a solution of Triton X-100 (0.1%), in a 1:10 ratio (w/v), by using a shaker under stirring at 200 rpm at 5.0°C for 40 min to

obtain the crude enzyme extracts (CEE). Before collecting the supernatant, the CEE passed a filter with gauze followed by centrifugation at 10,600 × *g* and 4.0°C for 10 min. To prevent the growth of contaminating microorganisms, we added sodium azide (final concentration of 0.02%) to the CEE and kept the enzymatic extracts at 4.0°C until use.

The commercial ascomycetes *Trichoderma reesei* (ATCC60787) and *Aspergillus aculeatus* (F-50 NBRC108796) were grown in a sterile liquid culture medium,[19] supplemented with 2.5% of SD to obtain CEE, and used as a reference for cellulolytic extracts production. Six 7.0 mm mycelial disks of *T. reesei* and *A. aculeatus*, pre-cultivated individually in PDA for 7 days, were used as inoculum, and 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. Once cultivation time finished, the samples underwent the same treatment reported before to obtain the CEEs for cocktail formulation to enzymatic hydrolysis essays.

2.5 | Hydrothermal pretreatment of empty fruit bunches (EB)

A mixture of 60 grams of EB and 710 ml of tap water underwent hydrothermal pretreatment (autohydrolysis) in a high/low-pressure reactor (4520 - Parr Instruments Company, Moline, Illinois USA) at 180°C for 40 min and constant stirring (600 rpm). After cooling and simple filtration for liquor removal, the now-called hydrothermally pretreated empty bunch (EB-H) had its moisture content determined and then stored in a closed container at 4.0°C.

2.6 | Enzymatic hydrolysis of EB-H using CEE of basidiomycetes, ascomycetes, and commercial enzymes (CE)

Two different enzyme mixtures underwent hydrolyses: (C1) CEE of basidiomycetes (12.69 ml g^{-1} of EB-H dry weight) with CEs Cellulase of T. reesei (ATCC 26921) (Celluclast®) (Sigma-Aldrich®) and cellobiase of A. niger (Novozyme–188®) (Sigma-Aldrich®), and (C2) CEE of basidiomycetes (12.69 ml g^{-1} of EB-H dry weight) and CEE of *T. reesei* and *A. aculeatus* ascomycetes. The former with the following protein dosage: 0.65% of 12.5 mg g^{-1} and 0.35% of 12.5 mg g^{-1}, respectively; and the later with protein dosages of 0.65% of 12.5 mg g^{-1} and 0.35% of 12.5 mg g^{-1}, respectively. The hydrolysis plates—24 deep-well plates with a solid load of 5% (dry matter) of EB-H and a final volume of 2.2 ml of buffer solution (sodium citrate/citric acid, 100 mM, pH 5.0)—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*, 4.0°C for 10 min, and the supernatant was collected and stored at −20°C.

2.7 | Determination of enzymatic activities

Determination of FPase activity followed the colorimetric method with miniaturized dinitro salicylic acid (DNS) proposed by Xiao et al.[20] Microassays determine endoglucanase (CMCase) and xylanase activities using carboxymethyl cellulose (CMC) 2% and xylan beechwood 2%, respectively—Sigma-Aldrich, St. Louis, MO, USA ([21] modified). The B-glucosidase activity was determined in a 96-well PCR plate, using cellobiose 15 mM as substrate (Sigma-Aldrich), glucose released was quantified on an ELISA plate using a commercial glucose oxidase–peroxidase (GOD-POD) kit (Bioclin®), and absorbance read at 505 nm. All tests were done in triplicate, and standard deviations were less than 10% of the mean.

Laccase activity determined through oxidation of ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt - Sigma-Aldrich[22] and readings taken every 5 s of reaction (ε ABTS = 36,000). Determination of total peroxidase activity done in 96-well ELISA plates[23] modified) (ε ABTS = 36,000).

Manganese peroxidase activity determined in 96-well ELISA plates for a reaction medium composed of 75 µl of SR reagent solution (100 µl of 0.1% phenol red, 100 µl of sodium lactate 250 mM, 200 µl of bovine albumin 0.5%, 50 µl of MnSO4 2 mM, and 50 µl of 2 mM H2O2 2 mM), 150 µl of sodium succinate 20 mM buffer pH 4.5 and 75 µl of enzymatic extract. Plates were incubated at 30°C for 5 min, and the reaction was interrupted with the addition of 6 µl of NaOH 2 M. Absorbance was read at 610 nm (ε phenol red = 44,600).

Lignin peroxidase activity was determined by a method based on the oxidation of the Azure B dye[24] and the total peroxidases activity by subtracting the activity value obtained in the assay from that obtained for laccase activity. All the enzymatic activities are in UI g^{-1} of the dry lignocellulosic substrate—UI defined as the international catalytic unit that refers to the amount of enzyme that catalyzes the conversion of 1 µmol of substrate per minute.

2.8 | Analytical methods

We determined the content of total soluble proteins in the crude extracts through the bicinchoninic acid method (BCA)[25] in ELISA plates according to the

TABLE 1 Taxonomic estimate of fungal basidiomycete isolates through comparison between the sequences obtained with the amplification of the ITS 1 and ITS 4 region and those present in databases

Strain	Genera/Species[a]		
Code	NCBI	CBS	BOLD Systems
BRM−055676	*Flavodon flavus*	*Flavodon flavus*	*Flavodon flavus*
BRM−063103	Nd	Nd	Nd
BRM−055675	*Fomes fasciatus*	*Fomes fasciatus*	*Fomes fasciatus*
BRM−062379	*Pleurotus* sp.	*Pleurotus* sp.	*Pleurotus* sp.
BRM−060007	*Trametes* sp.	*Pycnoporus* sp.	*Pycnoporus* sp.
BRM−062381	*Pycnoporus* sp.	*Pycnoporus* sp.	*Pycnoporus* sp.
BRM−050072	*Coprinus* sp.	*Coprinus* sp.	*Coprinus* sp.
BRM−060012	*Pleurotus* sp.	*Pleurotus* sp.	*Pleurotus* sp.

Abbreviation: Nd, Not determined.

[a]The described genera showed a similarity greater than 98% with isolates present in the databases.

protocol Sigma-Aldrich® commercial kit,and the values 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 SC-1 system.

Quantification of glucose and xylose, after enzymatic hydrolysis, was performed through high-performance liquid chromatography (HPLC) (Agilent Technologies), under the following conditions: Aminex® Column HPX-87H (300 × 7.8 mm, Bio-Rad, solution of H2SO4 (5 mM)) 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 to the theoretical maximum.[7]

3 | RESULTS AND DISCUSSION

3.1 | Selection of basidiomycetes

The selection of basidiomycetes in this study focuses on their potential to improve the digestibility of biomasses during enzymatic hydrolysis. In Figure S1 (Supplementary material), one can see the mycelial growth and vigor of all macro-basidiomycetes; in terms of colony diameter measurement in culture media containing enriched agar. BRM-063111 strain showed accelerated growth in all the evaluated media, reaching the maximum diameter in 3 days and low mycelial vigor, compared to BRM-055676 and BRM-060013, which colonized the entire plate with agar media after 7 days (Figures S2 and S3). The vigors of BRM-055676, BRM-063103, BRM-062377, BRM-060007, BRM-062381, BRM-055677, BRM-060012, and BRM-062454 were maximum for all evaluated media (Figures S2), indicating that there were sufficient carbon/nitrogen ratio and micronutrients available to promote microbial metabolism.

Basidiomycetes can naturally break down lignocellulosic materials. However, their behavior may vary depending on the composition of the substrate; due to the apparatus lignocellulolytic enzymes of each species.[5] Genera Flavodon and Pleurotus are excellent deconstructors of plant cell walls.[26] The use of distinct oil palm biomass to enrich the media with agar allowed the growth and vigor of the evaluated basidiomycetes. They also showed similar behavior when grown in PDA medium, used as control.

BRM-060007 (*Trametes* sp.) and BRM-062381 (*Pycnoporus* sp.) showed fast and vigorous growth in the tested media. These two genera contain species that produce ligninolytic enzymes, such as laccases.[27] Carbon sources, temperature, humidity, and oxygenation are critical factors for mycelial growth and fructification of basidiomycetes. Fungi present quite simple nutritional needs, as they can use carbon sources such as glucose, polysaccharides such as starch and cellulose, or aromatic compounds such as lignin.[28]

Fungi that showed the best growth and mycelial vigor on agar containing empty fruit bunches (EB), and sludge from palm oil decanter (SD) also had a positive growth rate when on axenic cultivations with the EB (SC-1) or a mixture of it and SD (SC-2). After 15 days of cultivation under the tested conditions, fungi with the highest linear growth in SC-1 were BRM-063103, *Flavodon flavus* BRM-055676, BRM-060008, and *Trametes* sp. BRM-060007. Modified SC-1 enabled the second screening of basidiomycetes for the growth capacity in residual palm oil lignocellulosic biomasses. Then, eight basidiomycetes were selected based on their growth capacity and mycelial vigor.

3.2 | Molecular identification of selected basidiomycetes

Taxonomic estimates of selected basidiomycetes are in Table 1. Six genera were identified: *Flavodon* sp., *Fomes* sp., *Pleurotus* sp., *Trametes* sp., *Pycnoporus* sp., and *Coprinus* sp. It was impossible to identify the BRM-063103 strain due to the quality of the sequences. The use of ribosomal markers is less prone to problems (flaws in the primer, absence of PCR products, and duplication of bands) during PCR amplification when compared to those that encode proteins.[29] Besides, the Fungi kingdom presents great phylogenetic diversity, and a single specific molecular marker for the entire Kingdom does not exist, requiring the use of two or more to achieve better accuracy in molecular identification.[30]

The taxonomic estimate obtained allowed us to know genera of these basidiomycetes from the Brazilian cerrado or purchased from fairs (edible mushrooms). These fungi can be used in research and the biological pretreatment of lignocellulosic biomasses processes to obtain value-added bioproducts, such as fermentable sugars soluble.

Identification of these fungi at the species level is possible with other markers, in addition to the ITS; for instance, primers that amplify part of the second major subunit of RNA polymerase (RPB2), β-tubulin genes, calmodulin, among others.[31] In parallel with identification through regions of DNA, recent years have seen remarkable evolution of genomic technologies, allowing access to the complete genomic sequencing of different organisms. Concerning basidiomycetes, sequenced genomes have revealed insights into the process of colonization and deconstruction of lignocellulose.[32]

3.3 | Production of crude enzyme extracts (CEEs) by basidiomycetes

Eight fungi selected based on their growth capacity in the culture media containing only EB underwent enzymatic activity quantification and application for lignocellulosic pretreatment (Figure 1). Biotic degradation of lignocellulosic biomass in nature is dynamic and influenced by biochemical, organism, and community. The biochemical factor is the structure of plant fiber and enzymatic characteristics. Regarding the organism, the growth kinetics and enzymatic expression are important and influenced by environmental factors. At the community level, the metabolism of each organism, structure, competition, succession, and diversity.[4,28]

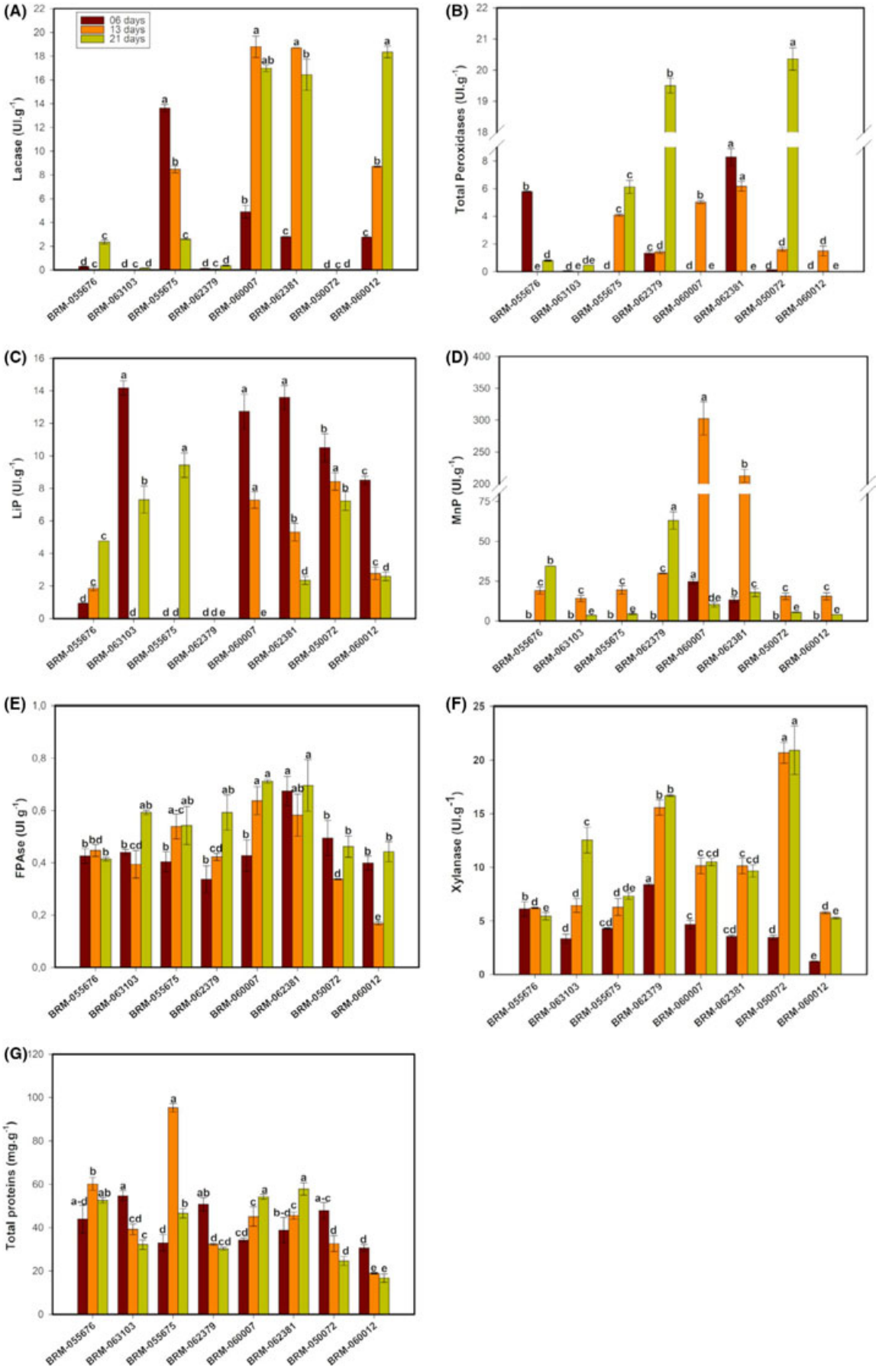
The highest laccase activity observed at 6 days of cultivation was with *Fomes fasciatus* BRM-055675, at 13 days with *Trametes* BRM-060007, and *Pycnoporus* sp. BRM-062381, and at 21 days with *Pleurotus* sp. BRM-060012 (Figure 1A). Regarding total peroxidase, *Pycnoporus* sp. BRM-062381 at 6 days, *Pleurotus* sp. BRM-062379 at 13 days, and *Coprinus* sp. BRM-050072 at 21 days gave the highest activity (Figure 1B). The best results for manganese peroxidase (MnP) were with *Trametes* sp. BRM-060007 at 6 and 13 days, and with *Pleurotus* sp. BRM-062379 at 21 days (Figure 1C). BRM-063103 at 6 days, *Coprinus* sp. BRM-050072 at 13 days, and *Fomes fasciatu*s BRM-055675 at 21 days showed the highest lignin peroxidase (LiP) activity (Figure 1D).

In the present study and the literature, ligninolytic activities varied according to sampling time and the species of macro-basidiomycete. López-Abelairas et al.[33] observed during the biological pretreatment of wheat straw under optimized conditions that the best activities of laccase (3 IU g^{-1}) and manganese peroxidase (2.5 IU g^{-1}) occurred at 21 days of *Pleurotus eryngii* cultivation, while the lower activities appeared at three and 5 days for *Irpex lacteus*. Inácio et al.[34] reported the highest laccase activity of *Pleurotus pulmonarius* at the 12th and 20th day of cultivation using orange residues, reaching 12.2 ± 0.15 IU ml^{-1}.

Laccase gene transcription in *Trametes velutina* 5930 can be induced by metal ions such as Cu^{2+} and Fe^{2+} and also by aromatic compounds structurally related to lignin (cinnamic acid, syringic acid, tannic acid, gallic acid, and guaiacol).[35] Besides, changes in cultivation conditions such as pH, temperature, incubation time, carbon/nitrogen ratio, and addition of mediators ($MnSO_4$, oxalate, ABTS, veratryl alcohol, and H_2O_2) directly influence the production of laccase, manganese peroxidase, and lignin peroxidase, as shown for *S. commune* IBL-06 grown in rice straw.[36]

Deconstructing enzymes of lignin and phenolic compounds are widely used and studied in the treatment of effluents, contaminated soils, clarification of drinks, modification of wood structures. Combined with cellulases and hemicellulases from *Trichoderma*, *Aspergillus*, *Penicillium*, and other fungi, they can improve saccharification of lignocellulosic materials.[37]

FIGURE 1 Enzymatic activities and total protein content of crude extracts (CEEs) of basidiomycetes grown in modified SC-1, at 28°C and 65% (±5) of moisture, at three cultivation times. Laccase (A); total peroxidases (B); lignin peroxidase—LiP (C); manganese peroxidase—MnP (D); FPase (E); xylanase (F); and total proteins (G). Different letters indicate statistically significant differences using the Tukey test ($p < 0.05$)


(A)
06 days
13 days
21 days
Lacase (UI.g-1)
(B)
Total Peroxidases (UI.g-1)
(C)
LiP (UI.g-1)
(D)
MnP (UI.g-1)
(E)
FPAse (UI g-1)
(F)
Xylanase (UI.g-1)
(G)
Total proteins (mg.g-1)
BRM-055676
BRM-063103
BRM-055675
BRM-062379
BRM-060007
BRM-062381
BRM-050072
BRM-060012

Total cellulase activities from the CEE of basidiomycetes obtained in this work are basal since basidiomycetes do not use primary sources to get this group of enzymes (Figure 1E). According to Elisashvili et al.,[28] there is a significant interdependence and overlap between ligninolytic and hydrolytic enzymes with the mechanisms that regulate them. However, they present many genes that encode other families of enzymes as accessory enzymes, which act in the degradation of cellulose and hemicellulose such as LPMO and proteins of the expansin-type, for example.[38] In addition, most cellulases investigated for industrial applications belong to filamentous fungi of the genera *Trichoderma*, *Penicillium*, *Fusarium*, *Humicola*, and others.[37]

The mechanisms that regulate the production of hydrolases in basidiomycetes are little known compared to ligninolytic enzymes. For hemicellulases, mainly xylanase, basidiomycetes exhibit higher activities since xylan is related to lignin and cellulose. A considerable number of xylanases commonly found in ascomycetes are present in basidiomycetes, including α and β-galactosidases, α-arabinofuranosidases, α-glucuronidases, acetyl-xylan esterases, a pectin-methyl esterase, and feruloyl esterases.[39]

Thus, sometimes these fungi secrete pools of xylanases to deconstruct hemicellulose, releasing sugar monomers and space in the fiber to reach cellulose/glucose. In this work, the highest xylanase activity observed at 6 days (8.38 ± 0.01 IU g^{-1}) occurred with *Pleurotus* sp. BRM-062379, and at 13 and 26 days with *Coprinus* sp. BRM-050072 and *Pleurotus* sp. BRM-062379, respectively (Figure 1F). According to González-Bautista et al.,[40] cellulase and xylanase activities depend on substrate composition. Premkumar et al.[41] reported the higher cellulase production by *Pleurotus djamor* during sugarcane bagasse fermentation and the increase in xylanase using wheat bran in the SC system. In another case, the use of a sequential solid/liquid fermentation, using corn cob as a substrate in the cultivation of *Pleurotus ostreatus*, resulted in higher endoglucanase (3.152 ± 139 IU L^{-1}) and xylanase (3.064 ± 40 IU L^{-1}) activities.[42] Coprinopsis cinerea cultured in rice straw SC showed an increase in xylanase activity after 6 days, reaching 0.57 IU ml^{-1} within 9 days and a gradual decrease until the 15th day.[43]

After solid cultivation, CEEs obtained from the SC-1 system that presented the highest content of total soluble proteins were BRM-063103 at 6 days, *Fomes fasciatus* BRM-055675 at 13 days, and *Pycnoporus* sp. BRM-062381 at 21 days (Figure 1G). The EB used as substrate presents the content of soluble proteins of 2.08% ± 0.12 (dry basis). The gain protein content can occur due to mycelial growth and secretion of enzymes.[44] Hence, biomass colonized by fungi becomes a source of soluble proteins, more specifically, deconstructing enzymes from the plant cell wall secreted during fungal metabolism.

3.4 | Production of crude enzyme extracts (CEEs) by ascomycetes

The content of total soluble proteins in the extracts of *T. reesei* and *A. aculeatus* throughout the cultivation period was verified (Figure 2A). After 7 days of cultivation, *T. reesei* extract showed the highest content of soluble proteins (6.64 mg ml^{-1}) compared to *A. aculeatus*. The characterization of the sludge from the palm oil decanter (SD), used as a supplement to the basal medium, indicated content of 15.44 ± 0.17 mg ml^{-1} of proteins, which explains the high levels of total soluble proteins observed at the beginning of the experiment.

Enzymatic profiles of ascomycetes were also determined (Figure 2B–E). FPase activity remained stable at *A. aculeatus* culture and for *T. reesei* showed an increase after 48 h. Xylanase activity increased over time for both fungi. *Trichoderma reesei* showed higher xylanase activity (3.32 IU mL^{-1}) than *A. aculeatus* (3.08 UI ml^{-1}). The higher endoglucanase activity was observed for *T. reesei* of 1.11 IU ml^{-1}, while *A. aculeatus* had 0.46 IU ml^{-1}. β-glucosidase activity profile was higher for *A. aculeatus* than *T. reesei*.

Among the cellulase-producing organisms, filamentous fungi belonging to the genera *Penicillium*, *Trichoderm*a, and *Aspergillus* are the ones that stand out the most, used on an industrial scale.[45] Within this context, *T. reesei* is a fungus with high productivity of different metabolites, including cellulases (endoglucanase) and hemicellulases (xylanase),[46]however, it presents low β-glucosidase activity. On the other hand, *A. aculeatus* is a better producer of the β-glucosidase enzyme,[37] which motivates its addition in cellulolytic cocktails aiming at biomass saccharification.[47] Therefore, SD is a potential additive to culture media to produce cellulases under the conditions studied in this work. This material presents 18.22% cellulose and can be a clean and low-cost alternative for cultivation.

3.5 | Hydrothermal pretreatment of empty fruit bunches (EB)

The purpose behind characterizing the combined use of EB biological and hydrothermal treatments was optimizing the production of enzymes and the release of sugars. The lignin of biomasses pretreated at high temperatures acts as a residual barrier, limiting enzymatic hydrolysis to obtain cellulosic ethanol.[35] Thus, the fact that the EB is subjected to sterilization removing the fruits (industrial

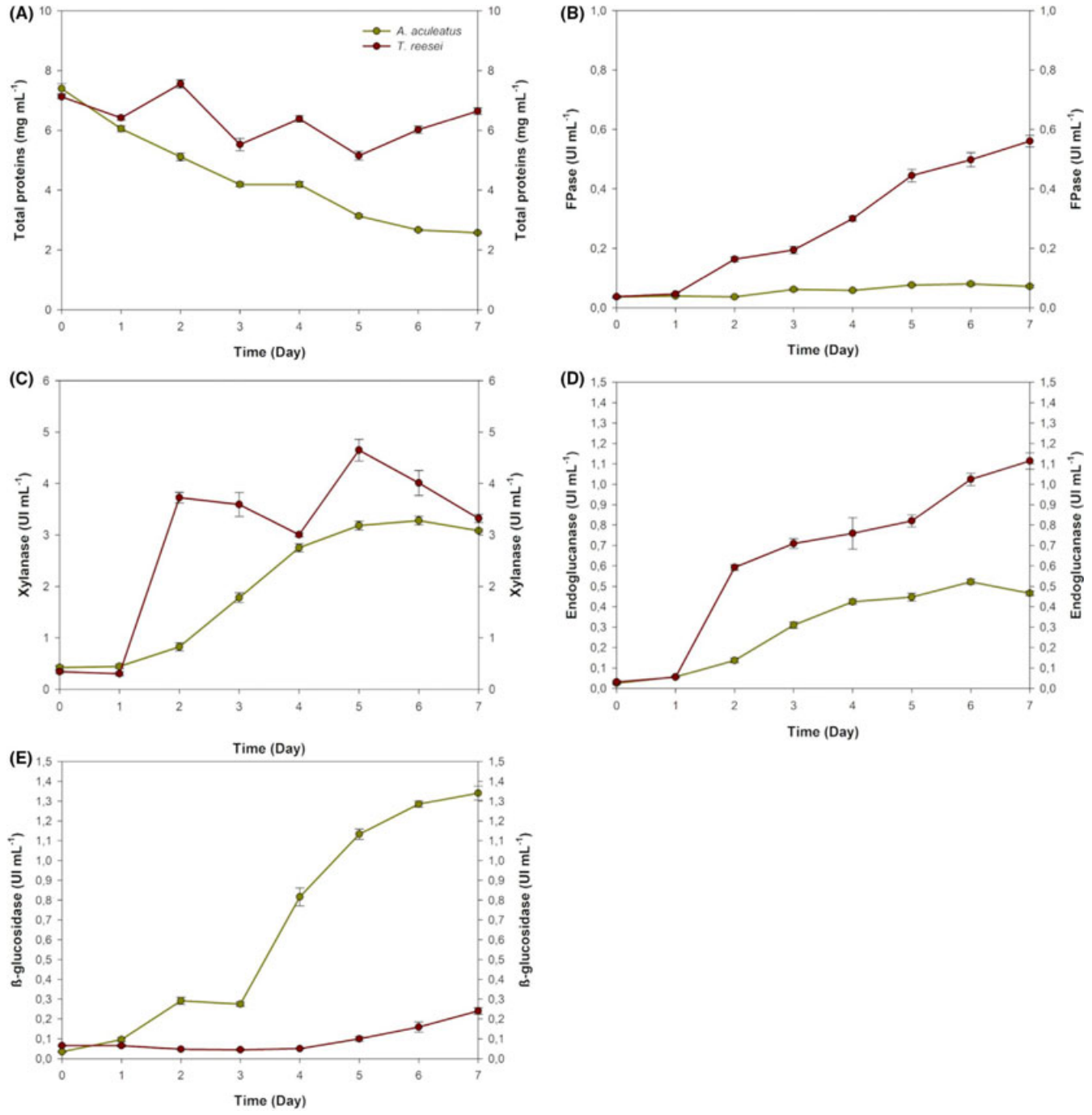


FIGURE 2 Profile of total proteins and holocellulolytic enzymes in the CEEs of ascomycetes *T. reesei* and *A. aculeatus* in submerged fermentation at 28°C and 150 rpm, culture medium supplemented with decanter sludge (SD). Total proteins (A); FPase (B); xylanase (C); endoglucanase (D); and β-glucosidase

process) and, subsequently, submitted to hydrothermal pretreatment (in this work), may have contributed to the collapse of the lignin, which can reduce the efficiency of the hydrolysis of the material under the tested conditions.

A part of the soluble lignin remained adsorbed on the fibers due to no EB-H wash. That can hamper the enzymatic hydrolysis. Besides, this material had a high lignin content, 42% (dry matter). Lignin plays a negative role in converting lignocellulosic biomass.[3] In this work, unwashed EB-H was used to verify the performance of the basidiomycetes extracts (with ligninolytic enzymes) in the presence of the lignin that eventually became adsorbed on the fibers of the bunches pretreated by autohydrolysis.

Some studies have applied wet oxidation pretreatment to break up the crystalline superstructure of EB and allow easier access of holocellulose by microbes.[48,49] Lee et al.[48] used oxygen concentrations of 3%–9% for 45 min and 180°C, and the pretreated EB was digested

in anaerobic conditions, increasing methane yield by 43%. Lee et al.[49] extend the scope of their previous work, including clarified manure in EB, which resulted in a 49% increase in methane production compared to the control.

Schmidt and Thomsen[50] reported that a temperature of 185°C was optimal for wheat straw fractionation, giving nearly three times more solubilized hemicellulose than at 150°C. Biswas et al.[51] achieved the maximum digestibility for pretreatment of forest residues at 190°C for 30 min with 7.5% oxygen loading. Both studies used similar conditions to ours (180°C for 40 min) in the pretreatment of lignocellulosic material, corroborating our findings of better sugar release with pretreated EB.

3.6 | Enzymatic hydrolysis of EB-H using cocktails of CEEs from basidiomycetes with commercial enzymes (CE) or CEEs from ascomycetes

According to the literature and previous experiments (data not shown), ascomycetes *T. reesei* and *A. aculeatus* produced cellulolytic enzymes in the submerged culture. The mix of CEEs from basidiomycetes and CE or CEEs from ascomycetes generated hydrolytic cocktails with ligninolytic and cellulolytic enzymes. Basidiomycetes are ligninolytic enzymes producers, and ascomycetes are sources of cellulases and hemicellulases. These enzymes aim to maximize sugar release during EB-H hydrolyses, and their mixture aims at increasing hydrolysis taxes because each enzyme will act in a specific substrate from biomass. Glucose and xylose yields were the response parameter, so the condition chosen for enzymatic hydrolysis favors breaking cellulose, even if the cocktail also has xylanase, laccase, and peroxidase. Despite not being the optimal conditions of all enzymes, because each one will have an optimal range of activity, they have acted as helpers of cellulases through their action on hemicellulose and lignin, facilitating the attack of cellulases on cellulose chains.

After EB-H enzymatic hydrolysis, glucose and xylose released by each cocktail combination were quantified (Figure 3). The results obtained using the combination of CEEs from basidiomycetes and CE (Celluclast® (Cellulase) and Novozyme-188 (Cellobiase) Sigma- Aldrich) demonstrated a higher glucose yield for the treatment [(CE) + *Flavodon flavus* BRM-055676], which differs statistically from the control (CE) (Figure 3A). The highest xylose yield came from the combination [(CE) + *Coprinus* sp. BRM-050072], which differs statistically from the control (CE) (Figure 3B). These results corroborate with Wang et al.,[7] which reported that basidiomycetes generate substances with synergistic action once combined with commercial enzymes during substrate colonization. In addition, inhibitors compounds from pretreatment did not reduce the hydrolysis efficiency, considering that the best sugar yield came from pretreated biomass (EB-H) (Figure 3).

Regarding the combinations of CEEs from basidiomycetes and ascomycetes, the highest glucose and xylose yields resulted from the combination [(Tr +Aa) + *Pleurotus* sp. BRM-062379] (Figure 3C–D). Studies that focus on the production of cellulases for industrial applications involve mainly filamentous fungi *T. reesei* and *A. aculeatus*.[37] According to Klein-Marcuschamer et al.,[47] low productivity and high cost of enzymatic cocktails are among the bottlenecks concerning hydrolysis of polysaccharides from lignocellulosic biomass and that could be more effective by creating a cocktail of enzymes derived from different fungi. The development and optimization of enzymatic cocktails have proved to be an area of intense research.[52]

Jung et al.[53] performed enzymatic hydrolysis of alkali-pretreated empty palm fruit bunches (5%, w/v) by extracts of *Penicillium* sp./TG2 and *T. reesei* RUT-C30 with 15 FPU g^{-1} solid, obtaining 7.4% and 40% hydrolysis yield of cellulose and hemicellulose, respectively. Elisashvili et al.[28] reported that what one expects in a rational screening of basidiomycetes is to find strains that produce synergistic mixtures of hydrolytic and oxidative enzymes. In an approach similar to the one used in the present study, Wang et al.[7] reported a synergistic effect of the enzymatic extract of the basidiomycete *Echinodontium taxodi* with a commercial enzyme, improving the hydrolysis of corn straw. At the conditions established in the present study, the positive effect promoted by the enzymatic extract of *Flavodon flavus* BRM-055676, compared to the other fungi together with CE, suggests the presence of enzymes that act synergistically with the enzymes Celluclast and Novozymes-188. The fact is that one can see an enzymatic increase provided by extracts of these fungi that needs further investigation. Also, the results were obtained with the CEE without any optimization process, only demonstrating its potential and influences at the established conditions.

In addition to ligninolytic and cellulolytic enzymes, some accessory enzymes secreted by macrofungi also facilitate lignocellulosic materials the hydrolysis.[38] LPMOs and expansins are examples of such accessory enzymes. There is still much to be studied and explored since many substances present in the crude extracts remain unknown and could influence the effectiveness of enzymatic cocktails.[7] Despite the advantage of a cocktail containing several classes of enzymes (ligninolytic and cellulolytic), some challenges ought to be faced, as each enzyme presents its thermal stability and an optimal pH range.[52]

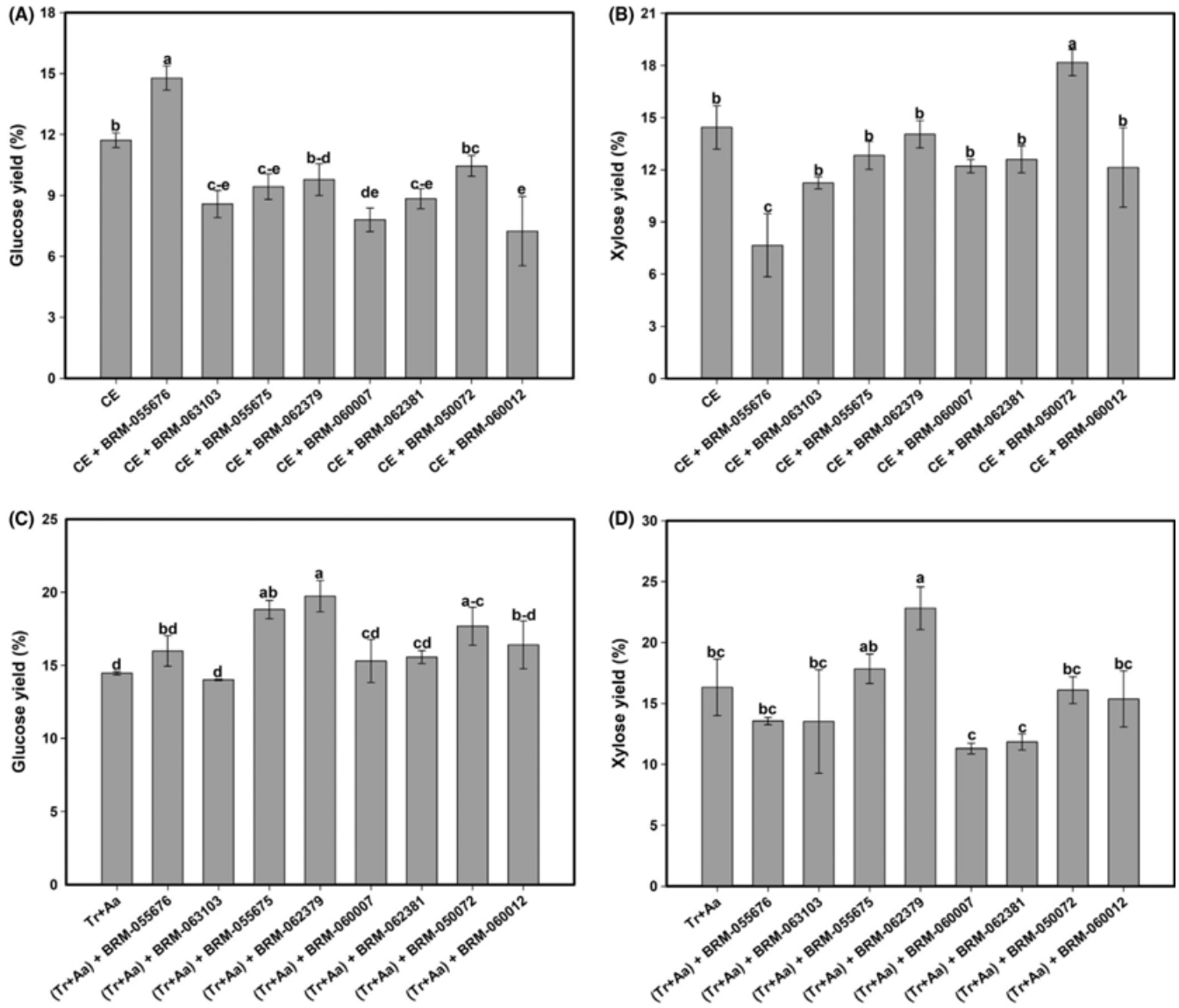


FIGURE 3 Yield (%) of sugars after hydrolysis of EB-H by cocktails C1 and C2 at pH 5.0, 50°C for 24 h. (A) glucose by C1, (B) xylose by C1, (C) glucose by C2 and (D) xylose by C2. C1 = CEE of basidiomycetes (12.69 ml g^{-1}) with CEs cellulase (0.65% 12.5 mg g^{-1}) and cellobiase (0.35% of 12.5 mg g^{-1}). C2 = CEE of basidiomycetes (12.69 ml g^{-1} substrate) and CEE of ascomycetes: *T. reesei* (0.65% of 12.5 mg g^{-1}) (Tr) and *A. aculeatus* (0.35% of 12.5 mg g^{-1}) (Aa). Data shown represent the average of analysis performed in triplicate from each essay also performed in triplicate. Different letters indicate statistically significant differences using the Tukey test ($p < 0.05$)

4 | CONCLUSIONS

Basidiomycetes strains from the Brazilian cerrado did grow in empty bunches of palm oil and produce enzymes. They were selected based on morphological aspects, enzymatic profile, and sugar yield during enzymatic hydrolysis by the cocktail. Fomes fasciatus exhibited growth rate and mycelial vigor during solid-state cultivation that extract combined with ascomycete extract resulted in a glucose yield of 19.4%. The cocktails that showed the best performance had a combination from *Pleurotus* sp. With *T. reesei* + *A. aculeatus* and *Flavodon flavus* with commercial enzymes, during hydrolysis of hydrothermally pretreated empty bunches, with glucose yield of 19.7% and 14.7%, respectively. These Brazilian basidiomycetes have great biotechnological potential to become commercially used strains for ligninolytic enzymes production.

ACKNOWLEDGEMENT

The authors acknowledge funding to E. A. S. from the Coordination for the Improvement of Higher Education Personnel (CAPES), a foundation within the Ministry of Education in Brazil, via the graduate program in plant biotechnology, Federal University of Lavras (UFLA).

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ETHICAL APPROVAL

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.

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SUPPORTING INFORMATION

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How to cite this article: da Silva EA, Mendes TD, Pacheco TF, et al. Colonization of oil palm empty fruit bunches by basidiomycetes from the Brazilian cerrado: Enzyme production. *Energy Sci Eng*. 2022;10:1189–1201. doi:10.1002/ese3.1093