


## Article

# Production of Enzymatic Extract with High Cellulolytic and Oxidative Activities by Co-Culture of *Trichoderma reesei* and *Panus lecomtei*

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**Abstract:** This work aimed to produce enzymatic fungi extracts with hydrolytic and oxidative activities to hydrolyze lignocellulosic biomasses efficiently. For this, the fungi *Trichoderma reesei* and *Panus lecomtei* were co-cultured using the vegetable biomasses oil palm decanter cake, wheat bran, and cottonseed cake as substrates in submerged fermentation. *T. reesei* and *P. lecomtei* showed partially compatible positive interaction on plates. The co-cultures respond positively to variations of temperature and inoculum interval, generating extracts responsible for higher hydrolysis yield when grown at 25 °C, and *P. lecomtei* is inoculated 24 h after *T. reesei*. The enzymatic extract production of co-cultures was also improved by modifying the components of the initial media and evaluating enzymatic activities, hydrolysis of sugarcane bagasse pretreated by autohydrolysis and ethanol production as a response. Five culture media were evaluated with variations in the composition of nutritional elements, minerals and substrates. The best extract showed a maximum cellulose hydrolysis efficiency of 68.7% compared with 44.8% of the initial medium. The ethanolic fermentation of hydrolysates obtained by co-culture extracts showed higher ethanol yields than monocultures. This work demonstrates the use of fungi co-cultures to produce enzymatic extracts composed of cellulolytic, hemicellulolytic, and ligninolytic enzymes complexes, which allow hydrolyzing pretreated lignocellulosic biomass with high efficiency, generating hydrolysates that are easier fermented by yeast.

**Keywords:** co-cultures; cellulases; laccases; ethanolic fermentation; *Trichoderma reesei*; *Panus lecomtei*



**Citation:** Peláez, R.D.R.; Wischral, D.; Cunha, J.R.B.; Mendes, T.D.; Pacheco, T.F.; Siqueira, F.G.d.; Almeida, J.R.M.d. Production of Enzymatic Extract with High Cellulolytic and Oxidative Activities by Co-Culture of *Trichoderma reesei* and *Panus lecomtei*. *Fermentation* **2022**, *8*, 522. <https://doi.org/10.3390/fermentation8100522>

Academic Editors:

Łukasz Łopusiewicz and  
Paweł Kwiatkowski

Received: 13 September 2022

Accepted: 6 October 2022

Published: 8 October 2022

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## 1. Introduction

The conversion of lignocellulosic biomass sugars into biofuels and chemicals comprises different process steps that have been optimized over the years [1]. Before the fermentation process, the lignocellulosic undergoes pretreatment and hydrolysis processes. First, it is necessary to make the cellulose fibers available for enzymatic hydrolysis. Cellulases are responsible for recognizing and degrading cellulose fibers, releasing glucose monomers. However, effective hydrolysis is necessary to modify hemicellulose fibers and lignin. Some types of enzymes work in synergy with cellulases, such as hemicellulases, ligninases, lytic polysaccharide monooxygenase (LPMO), and accessory proteins as expansins and swollenins [1]. Glucose, xylose, and other sugar monomers are produced in the process and used as carbon sources by fermenting organisms. In addition to sugars, the hydrolysate will contain organic acids, furaldehydes, and phenolic compounds, which are released and formed during the pretreatment and hydrolysis processes [2].

Consortiums or co-cultures, in addition to having the potential to increase the efficiency of enzymatic hydrolysis, could also serve to improve the hydrolysate fermentation

efficiency of organisms such as *Saccharomyces cerevisiae* by detoxifying inhibitors present in hydrolysates. *T. reesei* extracts were able to remove furaldehydes, acetic, and benzoic acids from hydrolysates, leading to increased ethanol production in the fermentation step [3]. On the other hand, oxidative enzymes such as laccase are related to the degradation of phenolic compounds. [4]. Thus, it is hypothesized that the use of hydrolysates generated with enzymatic extracts from cellulolytic and ligninolytic fungi could favor hydrolysis and fermentation processes.

A selective consortium of fungal strains with different genetic and phenotypic characteristics can add other enzymatic functions and increase its applications to biomass deconstruction to sugar [5,6]. The co-culture of different species of fungi allowed the generation of enzymatic extracts rich in various types of enzymes [7]. In previous work, we demonstrated that the co-culture of the ascomycete *T. reesei* RUT-C30 and the basidiomycete *P. lecomtei* produced in enzymatic extracts rich in cellulases, ligninases and hemicellulases, which allowed the increase in the hydrolysis yield of pretreated sugarcane bagasse [8].

This work aimed to improve the production of cellulolytic and oxidative enzymes by the co-culture of *T. reesei* RUT C30 and *P. lecomtei* to generate hydrolysates with high sugar content and less toxicity to the fermenting microorganisms.

## 2. Materials and Methods

### 2.1. Biomass and Fungi Strains

Oil palm decanter cake (OPDC) was kindly donated by Denpasa SA, Pará State—Brazil, was composed of cellulose 16.8%, hemicellulose 5.9%, lignin 17.4%, crude protein 15.3%, etheric extract 10.5% and ash 9.2%. Cottonseed and wheat bran were purchased from agricultural stores (Brasilia, Federal District, Brazil). These biomasses were firstly dried at 65 °C for two days and crushed using a micro-Wyllie type mill (diameter ≤ 2 mm). Sugarcane bagasse pretreated was obtained from Centro de Tecnologia Canavieira—CTC (São Paulo, Brazil). It was pretreated by autohydrolysis at 192 °C and 10 bar for 38 min in a high-pressure reactor and then called pretreated sugarcane bagasse (PSB).

*Panus lecomtei* BRM 044603 (Pl) belongs to the Collection of Microorganisms and Microalgae Applied to Agroenergy and Biorrefineries—Brazilian Agricultural Research Corporation (Embrapa Agroenergia), Brasília—DF, Brazil. *Trichoderma reesei* RUT-C30 ATCC 56,765 (Tr) was acquired from American Type Culture Collection (ATTC), Manassas, VA, USA. The *Saccharomyces cerevisiae* JP1 commercial strain, industrially used in bioethanol production in Brazil, was employed in the fermentation assays.

### 2.2. Growth Interaction on Solid Media

Pl was placed with Tr mycelium in potato dextrose (PDA) agar plates, to understand the compatibility of the fungal strains in vitro. They were separated on the plate by approximately 4 cm. Photographic records were kept for nine days, and various types of interaction analysis were performed according to Molla et al. [9] and Mohammad et al. [10]. This experiment was also performed to monitor pigment changes or structure formation in the interaction zone.

### 2.3. Selection of Temperature and Inoculum Time Interval for Tr and Pl Co-Cultures

For the selection of temperature and inoculum time of co-cultures, 50 mL of autoclave sterile modified Mandels & Weber medium [11] or initial medium (IM) (Table 1) with 1.25 g of OPDC (2.5% w/v) in 125 mL Erlenmeyer flasks. For monocultures, five mycelial discs (10 mm diameter each) of fully colonized agar of Pl or Tr were used as inoculum. In co-culture, five mycelial discs of fully colonized agar of Pl were inoculated. After 24, 48, and 72 h, each flask was inoculated with two mycelial discs of Tr. Cultivations were carried out at 25, 28, and 31 °C, 150 rpm for eight days with three biological replicates.

**Table 1.** Culture media composition for the optimization of enzyme extracts production by monocultures and co-cultures of PI and Tr.

Component	Initial Medium (IM)	M1	M2	M3	M4	M5	Unit
Urea	0.30	0.45	0.45	0.15	0.45	0.15	g/L
Yeast extract	0.75	1.13	0.38	0.38	0.75	1.13	g/L
Bacteriological peptone	0.25	0.13	0.13	0.13	0.13	0.13	g/L
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	1.40	2.10	2.10	2.10	2.10	0.70	g/L
KH <sub>2</sub> PO <sub>4</sub>	2.00	3.00	3.00	1.00	2.00	3.00	g/L
CaCl <sub>2</sub>	0.40	0.20	0.20	0.20	0.20	0.60	g/L
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.30	0.45	0.45	0.15	0.45	0.15	g/L
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.00	0.43	0.43	0.43	0.43	0.43	g/L
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	1.40	2.80	0.00	0.00	1.40	2.80	mg/L
CoCl <sub>2</sub> ·6H <sub>2</sub> O	2.00	0.00	0.00	0.00	0.00	4.00	mg/L
MnSO <sub>4</sub> ·H <sub>2</sub> O	1.04	0.00	2.08	2.08	1.04	0.00	mg/L
FeSO <sub>4</sub> ·7H <sub>2</sub> O	5.00	10.00	0.00	10.00	0.00	10.00	mg/L
Oil palm decanter cake (OPDC)	25.00	0.00	0.00	50.00	25.00	0.00	g/L
Cottonseed cake (CSC)	0.00	0.00	50.00	50.00	50.00	50.00	g/L
Wheat bran (WB)	0.00	20.00	20.00	20.00	20.00	20.00	g/L

#### 2.4. Selection of Media Composition for Enzyme Production in Submerged Fermentation

Five different media solutions (M1, M2, M3, M4, and M5) were evaluated, and they are detailed in Table 1. These media were established by altering or removing the concentrations of components of initial medium (IM) and adding wheat brand and cottonseed cake. Monocultures and co-cultures inoculum parameters were optimized as described in Section 2.3.

#### 2.5. Enzyme Extract Obtention and Activity Assays

After incubation, the cultures were transferred to 50 mL flasks for centrifugation, at  $10,600 \times g$  at 5 °C for 10 min, to separate residual biomass and fungal mass. The supernatant obtained was filtered through filter paper to remove suspended particles. This filtered was called crude enzymatic extract. FPase (total cellulases) and laccase (oxidative enzyme) were measured in the supernatant obtained. FPase was detected using Whatman® No. 1 filter paper as substrate, and the reducing sugar concentration was determined by the dinitrosalicylic acid (DNS) method at 540 nm [12,13]. Laccase was detected by oxidation of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) 5 mM at 420 nm [14,15]. All assays were performed using a UV-Vis spectrophotometer. An enzyme unit (U) was defined as an enzyme quantity that produces 1 µmol per minute.

#### 2.6. Hydrolytic Performance of Enzymatic Extracts

Enzymatic extracts obtained by mono or co-culture were tested for their ability to hydrolyze lignocellulosic biomass. Hydrolysis of pretreated sugarcane bagasse (PSB), composed by 35.5% cellulose, 16.1% hemicellulose, 34% lignin and 4.4% ash, was performed in 50 mL flasks. In each flask was added 1.75 g of PSB (5% (*w/v*), dry mass), enzyme extract of monocultures or co-cultures were fixed to 24.5 mL, and add 10.5 mL of 0.5 M citric acid/sodium citrate pH 5.0 was added. The flasks were closed by a rubber stopper and transferred to incubation at 50 °C, 200 rpm for 48 h. Novozymes Cellic® Ctec3 at 15 FPU per gram of PSB was used to compare the hydrolysis yield of monocultures and co-cultures, and an enzyme-free assay was used as a control. After the reaction, 0.5 mL aliquots were collected, placed in ice to stop the reaction and centrifuged at  $21,952 \times g$  for 5 min. The supernatants were quantified for glucose/xylose concentration analysis in High-Performance Liquid Chromatography (HPLC).

Hydrolysis efficiency (%) was calculated based on the conversion rate of cellulose and hemicellulose into glucose and xylose, respectively, using Equations (1) and (2) based on Soni et al. [16]:

$$\text{Hydrolysis efficiency of cellulose (\%)} = 100 \times [\text{Glucose}] \times [\text{Volume}] / (1.11 \times f \times [\text{Biomass}]) \quad (1)$$

$$\text{Hydrolysis efficiency of hemicellulose (\%)} = 100 \times [\text{Xylose}] \times [\text{Volume}] / (1.136 \times f \times [\text{Biomass}]) \quad (2)$$

where [Glucose or Xylose] is the concentration of glucose or xylose (g/L), [Volume] is the total volume in the enzymatic hydrolysis (0.035 L), 1.11 is the factor balance of mass balance of cellulose conversion to glucose, 1.136 is the factor balance of mass balance of hemicellulose conversion to xylose,  $f$  is the cellulose or hemicellulose fraction in dry mass (g/g) and [Biomass] is the dry mass of PSB used in the enzymatic hydrolysis (g/L). The maximum theoretical value of glucose obtained from cellulose was 20.96 g/L, while the maximum theoretical value of xylose obtained from hemicellulose was 8.88 g/L. Biomass characterization was determined by using the methodologies recommended by National Renewable Energy Laboratory (NREL) [17].

### 2.7. Fermentation of Hydrolysates by *S. cerevisiae*

*S. cerevisiae* strain JP1 cells were replicated on solid YPD (yeast extract 1%, peptone 2% and glucose 2%) medium. After growth, colonies were used to inoculate 100 mL of YPD in 250 mL flask, and subsequently incubated for 48 h at 30 °C and 120 rpm. Then, cells were pelleted by centrifugation, washed three times with sterile water and used as inoculum to ferment the hydrolysates. Yeast cell dry was determinate from 0.8 mL of pelleted yeast cells, deposited in glass plates, transferred to an oven at 70 °C for 24 h and measured until constant weight, expressed on a dry basis per mL.

Hydrolysates obtained from PSB, treated with monocultures and co-cultures enzymatic extracts, were used to evaluate glucose fermentation by *S. cerevisiae* strain JP1. Each hydrolysate was centrifuged at  $10,600 \times g$  for 10 min to separate PSB solid residual. Then, the liquid fraction of hydrolysate was filtered to remove suspended particles. When needed, the hydrolysates were supplemented with commercial glucose to obtain a minimum concentration of ~8.0 g/L, allowing for comparison of hydrolysates fermentability. 25 mL of each hydrolysate was sterilized in 50 mL flasks and inoculated with 1 g/L of *S. cerevisiae* strain JP1 cells, obtained as previously described. The flasks were incubated at 30 °C and 120 rpm for 7 h, 200 µL sample of each hydrolysate was obtained every hour for HPLC analysis.

The conversion of glucose into ethanol by *S. cerevisiae* JP1 was evaluated according to yield, efficiency and productivity, calculated by following equations:

$$\text{Yield (g/g)} = [\text{Ethanol}] / [\text{Glucose}] \quad (3)$$

$$\text{Productivity (g/L/h)} = [\text{Ethanol}] / [\text{Fermentation time}] \quad (4)$$

$$\text{Efficiency (\%)} = 100 \times [\text{Ethanol}_{\text{final}} - \text{Ethanol}_0] / (0.511 \times ([\text{Glucose}_0 - \text{Glucose}_{\text{final}}]) \quad (5)$$

where, [Glucose] and [Ethanol] are values of concentrations in g/L. [Fermentation time] is the time elapsed in the fermentation process until glucose is totally consumed (maximum time at 7 h). 0.511 g/g is the theoretical yield stoichiometric for the conversion of glucose to ethanol.

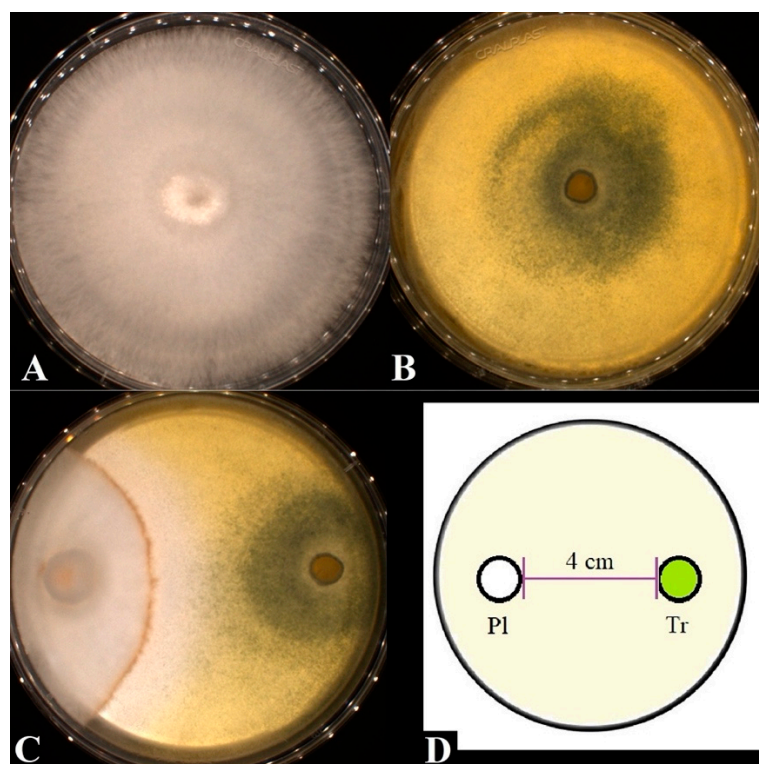
### 2.8. Analytical Methods

All experiments were carried out in biological and technical triplicate. The concentration of sugars (glucose, xylose and cellobiose), ethanol, glycerol, acetic and formic acids, hydroxymethylfurfural (HMF) and furfural, were quantified by HPLC, equipped with an HPX-87H column. The samples were analyzed with 5 mM H<sub>2</sub>SO<sub>4</sub> mobile phase, column temperature of 45 °C and flow rate of 0.6 mL/min. Calculations, Tukey test ( $p < 0.05$ ) and ANOVA were performed in Excel®, Statistica® 7.0 and SISVAR® software, respectively.

### 3. Results and Discussion

#### 3.1. Growth Interaction of Tr and Pl

Previously, it has been demonstrated that Tr and Pl are efficient cellulase and ligninolytic enzyme producers, respectively [8]. Pl and Tr growth patterns were evaluated individually and in interaction on PDA plates (Figure 1A–D). The growth pattern of Pl is characterized by the formation of a white cottony mass distributed concentrically in the plate area (Figure 1A). Tr turns medium yellow and produces green spores (Figure 1B). In the interaction, both strains grow in the medium without showing a zone of inhibition/deadlock, but stop their growth at a contact point (Figure 1C). According to Molla et al. [9], this is a pattern of partial mutual intermingling, so the strains are partially compatible. A brown-pigmented line is notable at approximately 1 cm from the area where both fungi converge, closer to the starting point of Pl growth (Figure 1C). This type of morphological change has been associated with a competitive phenomenon, where basidiomycete fungi defend themselves against antagonistic organisms, in response to chemical stress, through the release of oxidative enzymes, such as laccase, which lead to an accumulation of volatile and diffusible organic compounds (VOCs and DOCs), toxins, reactive oxygen species (ROS) and hydrolytic enzymes [18]. Pigmentation in interaction zones was associated with the release of interacting VOCs in plates with the antagonist *Trametes versicolor* and *Stereum gausapatum* [19] and with the release of laccases in the interaction of *Coprinus comatus* and *Trichoderma harzianum* [20]. Then, in the interaction of Pl and Tr, there are similar chemical changes in the medium in response to a competitive relationship, which may involve the secretion of laccase.

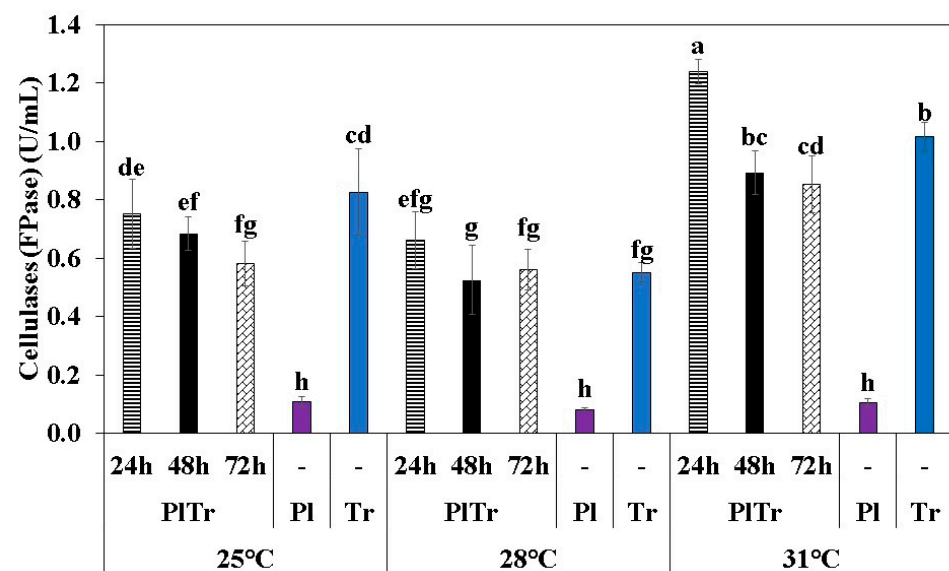


**Figure 1.** The growth pattern of *P. lecomtei* BRM 044603 (Pl) (A) and *T. reesei* ATCC 56765 (Tr) (B), and their growth interaction (C) on potato dextrose agar (PDA) plates after 8 days at 28 °C. The growth interaction was carried out by placing the mycelial disc of both fungi on the same plate, separated by 4 cm (D).

#### 3.2. Selection of Temperature and Inoculum Interval of Tr and Pl in Co-Cultures

In previous experiments by our group, co-cultures of *P. lecomtei* and *T. reesei* produced enzymatic extracts rich in laccase and peroxidative enzymes. These extracts enhanced

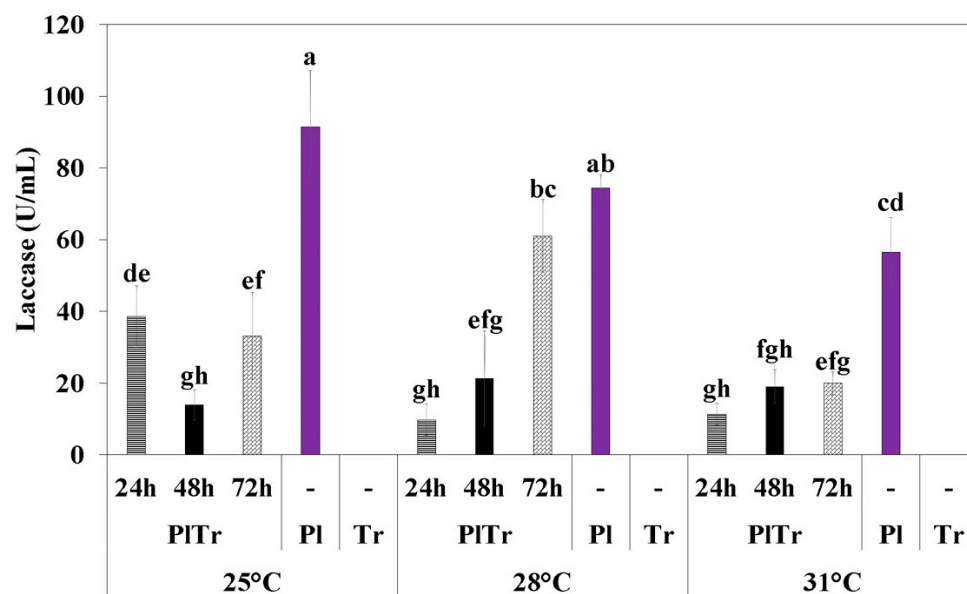
the release of sugars in sugarcane bagasse hydrolysis [8]. To produce an extract with better enzymatic hydrolysis performance, different incubation temperatures and inoculum intervals of Tr and PI were evaluated. Cellulase activities (FPase) were evaluated since high values are related to a higher bioconversion of cellulose into glucose monomers. The highest FPase activity was obtained in the extracts of Tr and PITr, carried out at 31 °C (Figure 2). There were significant differences only in the PITr treatment with an interval of 24 h and 31 °C (Tukey test,  $p < 0.05$ ).



**Figure 2.** Enzymatic activities of cellulases (FPase) obtained in submerged fermentation using Mandels & Weber medium with oil palm decanter cake (OPDC) as carbon source. The co-cultures were carried out by adding *T. reesei* (Tr) to 24, 48, and 72 h old monocultures of *P. lecontei* (PI). The experiments were carried out at three different temperatures, 25, 28, and 31 °C, for 8 days. The monocultures of Tr and PI were used as controls. Different letters over the bars mean significant differences ( $p < 0.05$ ) according to the Tukey test.

The cellulolytic enzyme production of PI was considerably low compared to PITr and Tr (Figure 2), regardless of the culture temperature. Similar results were obtained in Romero-Peláez et al. [8] in monocultures at 28 °C with the same medium composition. In general, white-rot fungi such as *Pleurotus*, *Trametes*, *Phanerochaete*, and *Pycnoporus* are known to secrete cellulases and hemicellulases less efficiently than soft-rot fungi (SRF) such as *Trichoderma*, *Aspergillus* and *Fusarium* [21–24], which could be evidenced in lower cellulase enzymatic activities in PI extracts.

Laccase activities were evaluated since they can contribute to enhancing bioconversion of cellulose into glucose monomers by modifying the lignin. The highest laccase activity was observed in PI at 25 °C ( $91.4 \pm 15.8$  U/mL) (Figure 3). In Šnajdr & Baldrian [25], it was shown that temperature variations in fungal cultures influence the secretion of oxidative enzymes such as laccase. In that study, submerged fermentation culture of *Pleurotus ostreatus* with cellulose as carbon source showed higher laccase production at higher temperatures, at 30 and 35 °C with 0.44 and 0.43 U/L. However, in the same study, *Trametes versicolor* showed higher laccase secretion (0.86 U/L) in the culture grown at 15 °C, which indicates that the effect of temperature on enzyme secretion also depends on the fungal species.



**Figure 3.** Enzymatic activities of laccases obtained in submerged fermentation using Mandels & Weber medium, with oil palm decanter cake (OPDC) as carbon source. The co-cultures were carried out by adding *T. reesei* (Tr) to 24, 48, and 72 h monocultures of *P. lecomtei* (PI). The experiments were carried out at three different temperatures, 25, 28, and 31 °C, for 8 days. The monocultures of *T. reesei* and *P. lecomtei* were used as controls. Different letters over the bars mean significant differences ( $p < 0.05$ ) according to the Tukey test.

Among the co-cultures, the PITr extract obtained at 28 °C and inoculum interval of 72 h showed the highest laccase value ( $61.1 \pm 10.1$  U/mL), without significant differences with the extracts from the PI monoculture treatments at 28 °C and 31 °C, but significantly different with the PI monoculture at 25 °C (Tukey test,  $p < 0.05$ ). The enzyme extracts produced by the co-cultures of PI and Tr showed different characteristics of enzymatic secretion of laccases and cellulases as a function of time between inoculum and temperature. Therefore, it was imperative to evaluate the potential of enzymatic hydrolysis using PSB to generate reducing sugars. Through co-cultures, it was still possible to obtain an enzymatic extract rich in Tr cellulases and PI laccases, which could favor the enzymatic hydrolysis of PSB.

### 3.3. Hydrolysis of Pretreated Sugarcane Bagasse Using Enzyme Extracts from Co-Cultures and Monocultures of Tr and PI

The crude enzyme extracts from each monoculture and co-culture treatment with Tr and PI were evaluated by enzymatic hydrolysis of PSB. The release of sugars and percentage of efficiency after 48 h of reaction is detailed in Table 2. The increase in cultivation temperature produced less efficient extracts in PSB hydrolysis. The highest efficiency of hydrolysis (44.7%) was found for the enzymatic extract of PITr, with an interval time of 24 h, incubated at 25 °C. The Tr monoculture at 28 °C showed similar efficiency to co-cultures of PITr at 25 °C (39.5%). This could indicate that the extracts obtained from the Tr monocultures and the PITr co-cultures, have a better hydrolysis yield at the lowest temperature evaluated, despite showing greater cellulolytic activity at 31 °C. In the literature, no information relates the temperature of the culture of similar *T. reesei* strains or their co-cultures with the efficiency of hydrolysis in a comparative way. However, this effect could be related to the fact that at 25 °C, there may be a greater secretion of cellulases that could act synergistically on the cellulose fragments of PSB, resulting in a higher glucose concentration. On the other hand, PI monocultures showed the worst hydrolysis efficiencies, regardless of temperature, since the fungus *P. lecomtei* secretes few cellulolytic enzymes measured in the FPase assay (Figure 2), and these enzymes probably do not recognize cellulose fragments from PSB

efficiently. Furthermore, the laccases of PI could contribute to modifying the lignin fraction of PSB, but there cannot degrade cellulose or hemicellulose.

**Table 2.** Glucose released and sugarcane bagasse hydrolysis efficiency of crude enzyme extracts of monocultures and co-cultures of *P. lecomtei* (PI) and *T. reesei* (Tr). The fungi were cultured in submerged fermentation using the Initial medium (IM), which contains oil palm decanter cake (OPDC). Enzymatic hydrolysis of sugarcane bagasse was carried out using 5% (*w/v*) of solids, during 48 h, 200 rpm at 50 °C. Different letters in the same column mean significant differences (*p* < 0.05) according to the Tukey test.

Extract Production Temperature	Culture	Inoculum Interval	FPU/g **	Glucose (g/L)	HE (%) *
25 °C	PITr	24 h	18.79	9.36 ± 0.48 <sup>b</sup>	44.74 ± 2.27 <sup>b</sup>
		48 h	17.09	8.61 ± 0.37 <sup>c</sup>	41.15 ± 1.78 <sup>c</sup>
		72 h	14.53	8.26 ± 0.60 <sup>c</sup>	39.51 ± 2.86 <sup>c</sup>
	PI	-	2.69	0.44 ± 0.04 <sup>g</sup>	2.09 ± 0.21 <sup>g</sup>
	Tr	-	20.66	6.00 ± 0.21 <sup>de</sup>	28.72 ± 1.01 <sup>de</sup>
28 °C	PITr	24 h	16.53	8.36 ± 0.54 <sup>c</sup>	39.99 ± 2.56 <sup>c</sup>
		48 h	13.13	6.54 ± 0.48 <sup>d</sup>	31.27 ± 2.28 <sup>d</sup>
		72 h	14.05	6.30 ± 0.63 <sup>de</sup>	30.11 ± 3.03 <sup>de</sup>
	PI	-	2.06	0.44 ± 0.04 <sup>g</sup>	2.10 ± 0.20 <sup>g</sup>
	Tr	-	13.79	8.28 ± 0.58 <sup>c</sup>	39.61 ± 2.76 <sup>c</sup>
31 °C	PITr	24 h	30.94	5.64 ± 0.56 <sup>e</sup>	26.97 ± 2.68 <sup>e</sup>
		48 h	22.31	4.12 ± 0.24 <sup>f</sup>	19.68 ± 1.13 <sup>f</sup>
		72 h	21.30	4.20 ± 0.42 <sup>f</sup>	20.11 ± 1.99 <sup>f</sup>
	PI	-	2.65	0.42 ± 0.03 <sup>g</sup>	2.02 ± 0.17 <sup>g</sup>
	Tr	-	25.4	5.83 ± 0.33 <sup>e</sup>	27.86 ± 1.57 <sup>e</sup>
Cellic Ctec 3			15.00	16.80 ± 0.48 <sup>a</sup>	79.80 ± 2.30 <sup>a</sup>

\* HE = Hydrolysis efficiency (%) = based on maximal glucose/xylose release in 5% (*w/v*) of PSB.

\*\* FPU/g = based on cellulase activity (FPU/mL) per 1.66 g of dry PSB.

Likewise, the culture temperature, and the inoculum time interval also affected the efficiency of PSB hydrolysis by the extracts. The co-cultures showed higher hydrolysis efficiency with an inoculum time interval of 24 h, regardless of temperature. The effect of the inoculum showed a greater relationship with cellulase activities, whose values were negatively affected by prolonging the interval time.

The co-culture strategy improved hydrolysis performance, but the inoculum interval and culture temperature influenced the enzymatic extract efficiency. Tr reached values slightly lower than the co-culture. This can be corroborated by comparing the maximum efficiency values among monocultures of PI (2.1%) and Tr (39.6%) with the efficiency value of glucose release among co-cultures (44.7%). Crude enzymatic extracts of microbial consortia have been used to enhance the release of fermentable sugars from enzymatic hydrolysis in various studies [26–28]. Co-culture studies using species of basidiomycetes and ascomycetes have been orientated towards sequential degradation strategies of lignocellulosic biomass. For example, in the biodelignification process, observed with *Phanerochaete chrysosporium* and *Trichoderma viride* [29], whose effect allows the increase of cellulose bioconversion using commercial enzymes. However, in Romero-Peláez et al. [8], the use of enzyme extracts from submerged PITS showed higher PSB hydrolysis efficiency than monocultures of Tr. Most of the consortia studies that focus on enhancing saccharification use SRFs as *Aspergillus* species, known to produce high amounts of cellulolytic and hemicellulolytic enzymes. This condition can improve the performance of hydrolysis when compared to the action of extracts produced by individual organisms. For example, the enzyme extract from co-culture of *A. flavus* and *A. penicillioides* enhanced the release of reducing sugars at 50–70% in hydrolysis of ionic-liquid pretreated bamboo when compared with their monocultures [30].

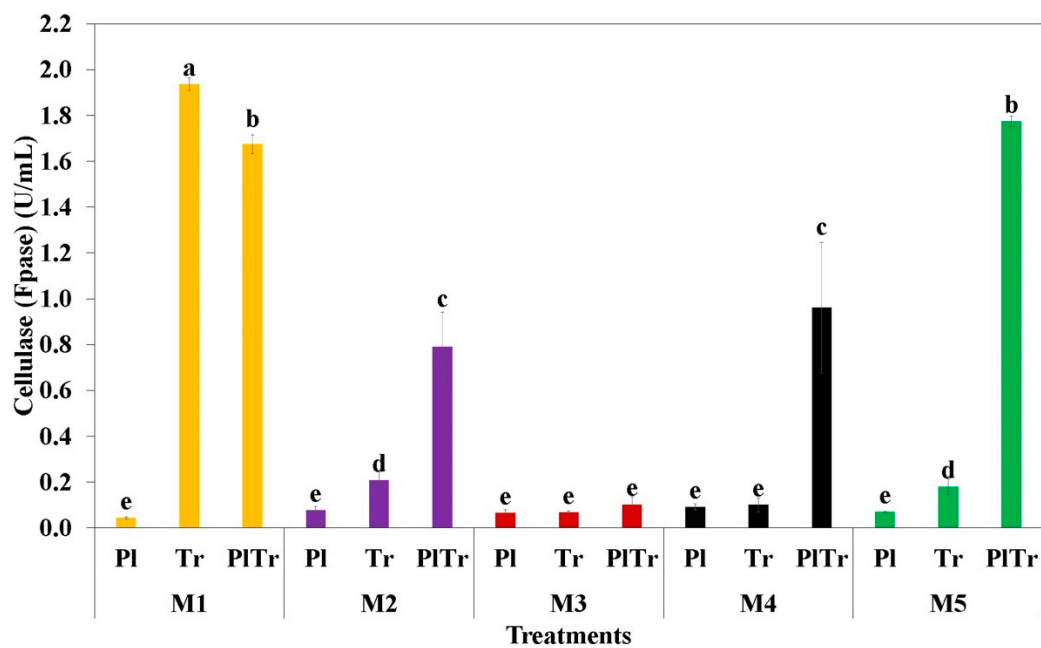
The crude extract of PI<sub>Tr</sub> with an inoculum interval of 24 h at 25 °C allowed the maximum hydrolysis efficiency of 44.7% to be obtained, and there was potential to improve the conversion. For this reason, the conditions for the production of this extract were chosen for subsequent experiments.

### 3.4. Culture Media for the Production of Lignocellulolytic Enzymes by Co-Cultures of Tr and Pl

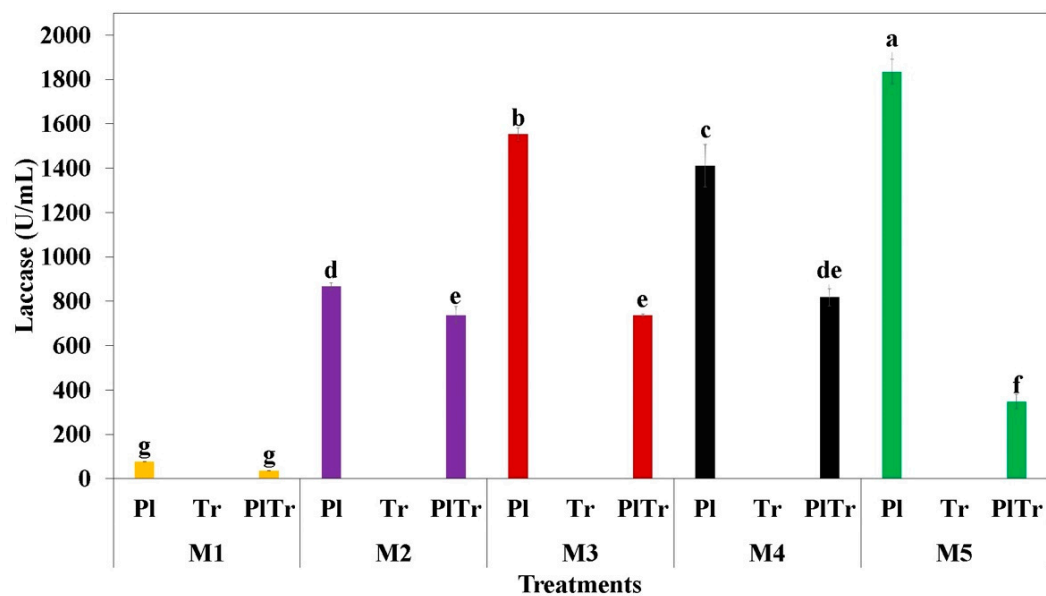
Five different culture media (M1, M2, M3, M4 and M5) were evaluated to improve the enzymatic production of the PI<sub>Tr</sub> co-culture. Those were obtained by varying the concentrations of IM components, adding the laccase-inducing component  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  [31], and the biomasses OPDC, cottonseed cake (CSC) and wheat bran (WB).

Tr and Pl were cultivated in monoculture and co-culture with an inoculum interval of 24 h at 25 °C to evaluate the effect of medium composition in the production of lignocellulolytic enzymes. After eight days, culture supernatants were collected and assayed for enzyme activities. Among the cellulase activities (FPase), the maximum values were obtained in cultures carried out with the M1 medium, with  $1.94 \pm 0.03$  U/mL in the Tr extract and  $1.67 \pm 0.04$  U/mL in the PI<sub>Tr</sub> extract (Figure 4). The distinctive characteristic of the M1 medium was the use of WB as a substrate and the absence of  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  and  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ . In the medium M5, the extract obtained in the PI<sub>Tr</sub> co-culture showed a cellulase activity value similar to the M1 ( $1.77 \pm 0.02$  U/mL U/mL). However, Tr monoculture showed very low activity. The main differences between M1 and M5 are the presence of CSC and  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , which may affect cellulase production by Tr in monoculture. In M5, WB and CSC were used as substrates in the same proportion as in M2. However, with the M2 medium, the cellulase activity of the PI<sub>Tr</sub> was lower ( $0.79 \pm 0.1$  U/mL). Thus, the differences among FPase activities can be related to the concentration of components in each medium as ions and nitrogen. The M4 medium induced the release of  $0.96 \pm 0.2$  U/mL of cellulase activity from the PI<sub>Tr</sub> co-culture, which did not significantly differ from the PI<sub>Tr</sub> extract from M2. The cultures of the M3 medium obtained the lowest cellulase values ( $0.07 \pm 0.1$  U/mL), which coincides with being the medium with the highest substrate load (120 g/L), using the three evaluated biomasses. Overall, the different combinations in the chemical components and the lignocellulosic biomasses evaluated favored the production of cellulases by PI<sub>Tr</sub> co-cultures. Only the Tr in the M1 medium had a higher value than the co-cultures. On the other hand, cellulase production is not increased by offering fungi different types of biomasses as carbon sources since the highest values were obtained only with WB. Additionally, in both the M1 and M5 media, the yeast extract was higher level (1.13 g/L), which indicates that this concentration stimulates the production of cellulases; this effect has already been demonstrated in previous studies [32].

The monocultures and co-cultures showed a different profile to laccase activity. First, except by the M1 medium, all the media lead to higher laccase activities in Pl monocultures. This is more evident in the M5 medium, where the extract from the Pl monoculture had higher laccase activity ( $1834 \pm 54$  U/mL) (Figure 5), which means 18-fold the laccase activity observed in the maximum value in Figure 3. There were no significant differences between the extracts from the PI<sub>Tr</sub> co-cultures with the M2, M3 and M4 media (736–818 U/mL). The M1 medium had the lowest value of laccase activity, indicating the importance of different substrates for the induction of this activity, in contrast to the production of cellulases. It is important to note that, unlike the M1 medium, the different combinations of chemical components and lignocellulosic biomasses increased the production of laccases in monocultures and co-cultures, which means that the strategy of optimization was successful in improving the secretion of laccases.



**Figure 4.** Enzymatic activities of cellulases (FPase) obtained in submerged fermentation using modified Mandels & Weber medium, with oil palm decanter cake (OPDC), wheat bran (WB), and cottonseed cake (CSC) as the carbon source. The media (M) composition is described in Table 2. Legends: *P. lecomtei* monoculture (PI), *T. reesei* monoculture (Tr) and *P. lecomtei* + *T. reesei* co-culture (PITr). Different letters over the bars mean significant differences ( $p < 0.05$ ) according to the Tukey test.



**Figure 5.** Enzymatic activities of laccase obtained in submerged fermentation using the media M1, M2, M3, M4 and M5, which contains oil palm decanter cake (OPDC), wheat bran (WB) or/and cottonseed cake (CSC) as substrate. Legends: *P. lecomtei* monoculture (PI), *T. reesei* monoculture (Tr) and *P. lecomtei* + *T. reesei* co-culture (PITr). Different letters over the bars mean significant differences ( $p < 0.05$ ) according to the Tukey test.

### 3.5. Hydrolysis of Pretreated Sugarcane Bagasse Using Crude Enzyme Extracts from Cultures Subjected to Variation in Medium Composition

The selected media allowed higher FPase and laccase activities in the extracts of monoculture and co-culture of Pl and Tr. As the individual activities do not correlate directly with biomass hydrolysis, the saccharification efficiency of the crude extracts obtained from monocultures and co-cultures in the media M1, M2, M3, M4 and M5 was evaluated as a function of the release of glucose and xylose in enzymatic hydrolysis of PSB. Table 3 summarizes the concentration (g/L) and the hydrolysis efficiency (%). The enzymatic extracts of PlTr showed higher efficiency of hydrolysis when compared to the extracts from monocultures. The maximum glucose value was observed with the PlTr extract obtained in the M4 medium, with 14.24 g/L, which corresponds to 67.86% efficiency in cellulose conversion, while the commercial enzyme Cellic Ctec 3 obtained 79.48% efficiency. In the literature, approximate values of hydrolysis efficiency in sugarcane bagasse pretreated by autohydrolysis have been reported. For example, Brar et al. [33] compared the hydrolysis efficiency of autohydrolytically PSB at 10, 15 and 20% (*w/v*) solids loading using an enzyme cocktail of Novozyme Cellic Ctec 2 with purified xylanase, with hydrolysis efficiencies of 61.9, 61.2 and 58.0%, respectively. Although the solid load used experimentally was <10% (*w/v*), and it was expected that at lower loads, the cellulose conversion could increase [34], the results show that the extract from the co-culture in the M4 medium has excellent commercial potential for the saccharification of pretreated biomass.

**Table 3.** Glucose/xylose released and hydrolysis efficiency of crude enzyme extracts of monocultures and co-cultures of *P. lecontei* (Pl) and *T. reesei* (Tr). The fungi were cultured in submerged fermentation using media M1, M2, M3, M4 and M5, which contains oil palm decanter cake (OPDC), wheat bran (WB) and/or cottonseed cake (CSC) as carbon source. Enzymatic hydrolysis of pretreated sugarcane bagasse (PBS) was carried out using 5% (*w/v*) of solids, during 48 h, 200 rpm at 50 °C. Different letters in the same column mean significant differences (*p* < 0.05) according to the Tukey test.

Culture Medium	Enzyme Extract	** FPU/g	Glucose (g/L)	* HE (%)	Xylose (g/L)	* HE (%)	Total HE (%)
M1	PlTr	25.21	9.61 ± 1.19 <sup>d</sup>	45.84 ± 0.05 <sup>d</sup>	5.53 ± 0.45 <sup>b</sup>	62.43 ± 5.03 <sup>b</sup>	50.87
	Pl	0.65	0.77 ± 0.09 <sup>h</sup>	3.64 ± 0.42 <sup>h</sup>	0.00 ± 0.00 <sup>f</sup>	0.00 ± 0.00 <sup>f</sup>	2.57
	Tr	29.16	9.90 ± 0.61 <sup>d</sup>	47.17 ± 2.90 <sup>d</sup>	0.80 ± 0.11 <sup>d</sup>	9.05 ± 1.20 <sup>d</sup>	35.96
M2	PlTr	11.92	9.20 ± 0.01 <sup>d</sup>	43.84 ± 0.05 <sup>d</sup>	6.21 ± 0.87 <sup>ab</sup>	70.14 ± 9.78 <sup>ab</sup>	51.79
	Pl	1.17	0.82 ± 0.10 <sup>h</sup>	3.93 ± 0.49 <sup>h</sup>	0.46 ± 0.10 <sup>ef</sup>	5.18 ± 1.09 <sup>ef</sup>	4.31
	Tr	3.12	6.74 ± 0.72 <sup>e</sup>	32.11 ± 3.45 <sup>e</sup>	0.73 ± 0.12 <sup>ef</sup>	8.29 ± 1.35 <sup>ef</sup>	25.11
M3	PlTr	1.54	1.26 ± 0.01 <sup>gh</sup>	6.00 ± 0.05 <sup>gh</sup>	3.81 ± 0.11 <sup>c</sup>	43.04 ± 1.27 <sup>c</sup>	17.04
	Pl	1.00	2.39 ± 0.11 <sup>g</sup>	11.39 ± 0.54 <sup>g</sup>	0.09 ± 0.06 <sup>ef</sup>	0.99 ± 0.73 <sup>ef</sup>	8.33
	Tr	1.02	4.56 ± 0.59 <sup>f</sup>	21.73 ± 2.82 <sup>f</sup>	0.15 ± 0.00 <sup>ef</sup>	1.74 ± 0.00 <sup>ef</sup>	15.85
M4	PlTr	<b>14.47</b>	<b>14.24 ± 0.20<sup>b</sup></b>	<b>67.86 ± 0.96<sup>b</sup></b>	<b>5.44 ± 0.11<sup>b</sup></b>	<b>61.44 ± 1.22<sup>b</sup></b>	<b>66.14</b>
	Pl	1.39	0.78 ± 0.03 <sup>h</sup>	3.74 ± 0.15 <sup>h</sup>	0.29 ± 0.12 <sup>ef</sup>	3.29 ± 1.35 <sup>ef</sup>	3.61
	Tr	1.52	4.72 ± 1.01 <sup>f</sup>	22.48 ± 1.01 <sup>f</sup>	0.13 ± 0.09 <sup>ef</sup>	1.50 ± 1.03 <sup>ef</sup>	16.30
M5	PlTr	26.71	11.64 ± 0.05 <sup>c</sup>	55.48 ± 0.26 <sup>c</sup>	6.42 ± 0.09 <sup>a</sup>	72.53 ± 0.99 <sup>a</sup>	60.71
	Pl	1.04	0.72 ± 0.04 <sup>h</sup>	3.44 ± 0.18 <sup>h</sup>	0.19 ± 0.08 <sup>ef</sup>	2.14 ± 0.86 <sup>ef</sup>	3.07
	Tr	2.70	4.06 ± 0.07 <sup>f</sup>	19.34 ± 0.34 <sup>f</sup>	0.58 ± 0.20 <sup>ef</sup>	6.56 ± 2.31 <sup>ef</sup>	15.60
Cellic Ctec 3		<b>15.00</b>	<b>16.68 ± 0.45<sup>a</sup></b>	<b>79.80 ± 2.30<sup>a</sup></b>	<b>6.06 ± 0.17<sup>ab</sup></b>	<b>68.48 ± 2.92<sup>ab</sup></b>	<b>76.43</b>

\* HE = Hydrolysis efficiency (%) = based on maximal glucose/xylose release in 5% (*w/v*) of \*\* FPU/g = based on cellulase activity (FPU/mL) per 1.66 g of dry PSB.

As well as the optimization results of temperature and inoculum time interval, the extracts that showed values in the intermediate ranges of laccase and cellulase, also obtained better hydrolysis efficiency. Important enzymes in lignocellulose bioconversion were most likely secreted in these types of extracts and could not be observed in the enzymatic assays.

Although the crude extract of PITr obtained in the M4 medium did not obtain the highest value of total cellulase activity ( $0.96 \pm 0.2$  U/mL), it was the best performance in glucose release. In addition to the action of cellulases and laccases, some enzymes that were not measured in the investigation, such as xylanases, arabinases, polygalacturonase, lignin peroxidases or auxiliary enzymes such as Lytic polysaccharide monooxygenases (LPMO) could be secreted in the cultures and have positive effects on the bioconversion of PSB. According to the results, the co-culture strategy, when the organisms are compatible, was advantageous to obtaining crude extracts with an adequate balance of lignocellulolytic activity for biomass conversion, which allows the modification and degradation of the structural components of the cell wall.

There is evidence of the importance of laccase enzyme activity in improving enzymatic hydrolysis [35,36]. Chabl -Villacis [36] observed that adding *Trametes hirsuta* extracts rich in laccases in the hydrolysis of pineapple leaf waste using commercial cellulolytic enzymes allowed a 10% increase in the cellulose conversion yield. However, there is also evidence of the inhibitory action of laccase in biomass hydrolysis Rocha-Martin [37]. In this case, it was observed a reduction of the hydrolysis yield between 22–42% when commercial *Myceliophthora thermophila* laccases were added simultaneously and sequentially in the hydrolysis of sugarcane bagasse mediated by commercial cellulases. In our case, the positive effect of laccases could be explained since their presence is not a supplement or external addition to a cocktail with cellulases, but instead that they are produced in the same batch together with the cellulase-producing microorganisms in co-culture systems.

On the other hand, the co-cultures had higher values of xylose release, regardless of the medium. The maximum value of xylose was obtained in the PITr extract in the M5 medium, with 6.42 g/L of xylose, or 72.53% conversion of hemicellulose. The removal of hemicellulose fragments in the PSB may be related to increased secretion of various hemicellulolytic enzymes or xylanases [38]. This removal allows the enzymes to have greater access to the cellulose fragments to be degraded. The relationship between the removal of hemicellulose and the increase in the performance of enzymatic hydrolysis has been evidenced in various studies [39,40]. PITr crude enzymatic extracts also have the potential to obtain xylose, which has multiple biotechnological applications as substrate in physical or biological processes to obtain furfural, xylitol, xylonic and xylaric acid, lactic acid, or 2G ethanol [41–44].

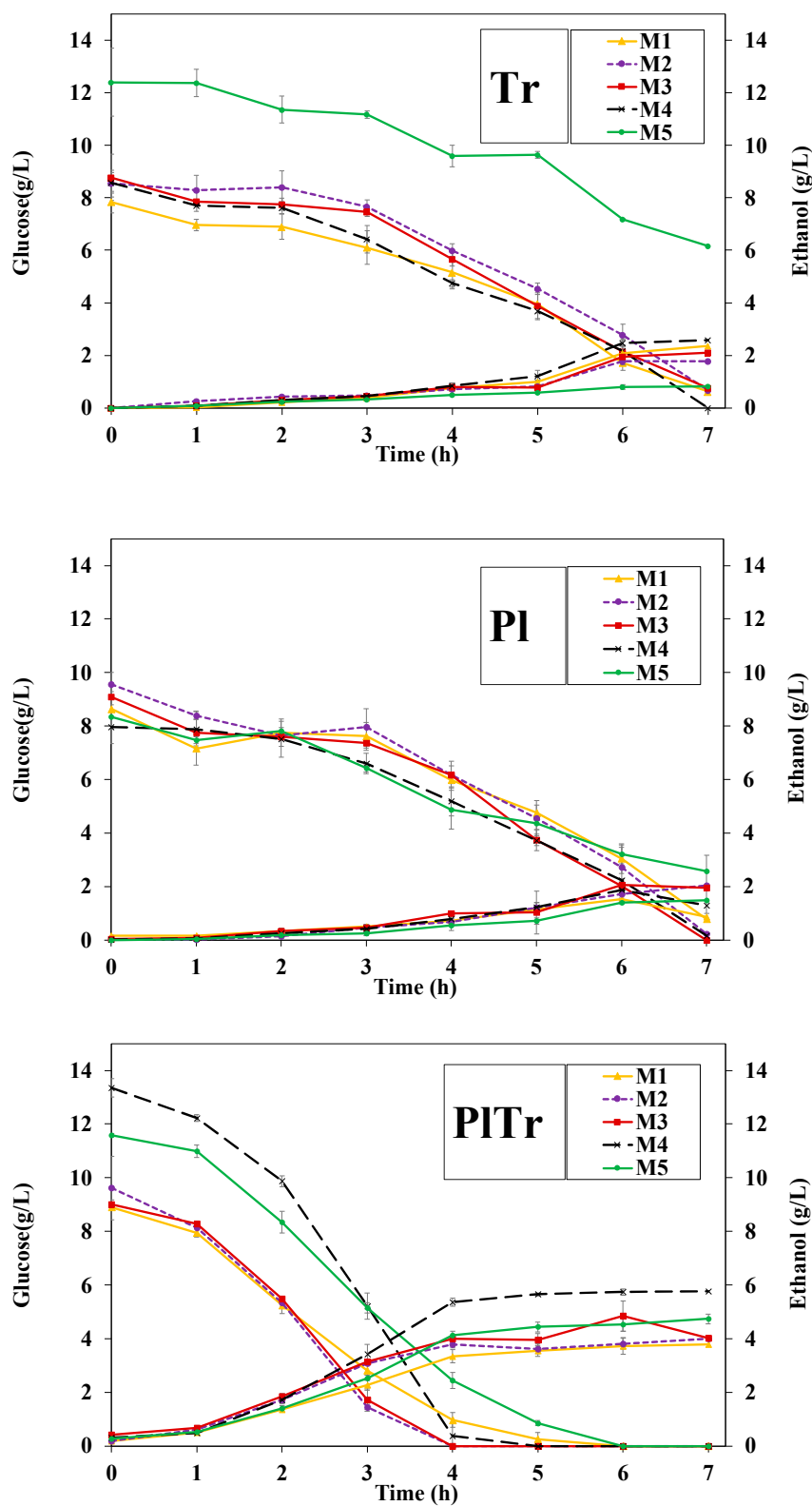
### 3.6. Fermentative Profile of Ethanol Production by *S. cerevisiae* from Hydrolysates of Pretreated Sugarcane Biomass

In addition to sugars, the lignocellulose-derived hydrolysates contain a series of compounds, such as acetic acid, furaldehydes, and phenolic compounds, that may affect the microorganism used in the fermentative step [45]. The hydrolysates obtained after the sugarcane bagasse's enzymatic hydrolysis, using the extracts from Tr, Pl and PITr in M1, M2, M3, M4 and M5 media, were used as fermentation substrate for *S. cerevisiae* JP1. The results of the fermentative kinetics are given in Figure 6 and Table 4. *S. cerevisiae* JP1 was able to metabolize glucose from PSB hydrolysate, but as expected, it was not able to metabolize xylose due to the lack of a functional xylose-consuming pathway [46].

The fermentation kinetics varied significantly among the different conditions, with the hydrolysates obtained by extracts of the PITr co-cultures showing the best conversion efficiencies (Figure 6). Indeed, the yeast was able to consume all the glucose in the co-culture hydrolysates after 4 h (media M2, M3 and M4) and 6 h (media M1 and M5). In comparison, it took around 7 h in the hydrolysates obtained by extracts of monocultures (Figure 6). The best glucose consumption in the hydrolysates obtained by extracts of the PITr resulted in higher ethanol production too. In these hydrolysates, the ethanol yields were around  $0.43 \text{ g} \cdot \text{g}^{-1}$ , whereas it was below  $0.30 \text{ g} \cdot \text{g}^{-1}$  in the other hydrolysates (Table 4).

The best performance was observed in the hydrolysate obtained by the PITr extract cultivated in the M4 medium, with a productivity of 0.82 and a yield of 0.43 (efficiency 79.94%). In the case of hydrolysates from monocultures, the best yeast performances were

obtained in the hydrolysates obtained by Tr cultured in the M1 medium (63.99%), and PI cultured in the M5 medium (49.97%) (Table 4).



**Figure 6.** Profile of glucose consumption and ethanol production by *Saccharomyces cerevisiae* from hydrolysates obtained by hydrolysis of PSB, using extracts from monocultures of *P. lecontei* (PI), *T. reesei* (Tr), and its co-culture (PITr) obtained in cultures with the media M1, M2, M3, M4 and M5.

**Table 4.** Glucose and ethanol concentration (g/L), yield (g/g), productivity (g/L/h) and fermentation efficiency (%) of hydrolysates, obtained by hydrolysis of PSB using extracts from monocultures of *P. lecomtei* (Pl), *T. reesei* (Tr), and its co-culture (PlTr) obtained in cultures with the media M1, M2, M3, M4 and M5.

Medium	Culture	Glucose (g/L)	Acetic Acid (g/L)	Ethanol (g/L)	Yield (g/g)	Productivity (g/L·h)	Efficiency (%)
M1	PlTr	8.91	1.28	3.79	0.43	0.63	79.44
	Pl	8.63 <sup>Δ</sup>	0.23	0.85	0.10	0.12	17.17
	Tr	7.83 <sup>Δ</sup>	0.46	2.36	0.30	0.34	63.99
M2	PlTr	9.61	1.46	3.99	0.41	1.00	77.66
	Pl	9.56 <sup>Δ</sup>	0.26	2.03	0.21	0.29	42.50
	Tr	8.54 <sup>Δ</sup>	0.63	1.77	0.21	0.25	43.96
M3	PlTr	9.00 <sup>Δ</sup>	1.09	4.01	0.45	1.00	78.33
	Pl	9.08 <sup>Δ</sup>	0.18	1.94	0.21	0.28	41.18
	Tr	8.77 <sup>Δ</sup>	0.45	2.11	0.24	0.30	51.58
M4	PlTr	13.35	1.17	5.76	0.43	1.44	79.94
	Pl	7.97 <sup>Δ</sup>	0.21	1.29	0.16	0.18	32.05
	Tr	8.57 <sup>Δ</sup>	0.38	2.58	0.30	0.37	58.81
M5	PlTr	11.59	1.29	4.74	0.41	0.79	75.57
	Pl	8.34 <sup>Δ</sup>	0.16	1.47	0.18	0.21	49.97
	Tr	12.40 <sup>Δ</sup>	0.49	0.82	0.07	0.12	25.69

Note: <sup>Δ</sup> Hydrolysates supplemented with commercial glucose.

One of the main drawbacks in converting sugars to ethanol in lignocellulosic hydrolysates is the formation of fermentation inhibitors during the pretreatment process or enzymatic hydrolysis [2]. In the case of PSB, furaldehydes such as hydroxymethylfurfural (HMF) or furfural, which negatively influence ethanolic fermentation, were not detected (data not shown). The concentration of acetic acid (1.09–1.46 g/L) detected in the hydrolysates from the hydrolysis of PSB by the extracts of the PlTr co-cultures was not high enough to inhibit yeast performance (Table 4). These results are in agreement with a previous study, which showed that different *Saccharomyces* strains, including JP1, tolerate acetic acid concentrations up to 5 g/L without significantly affecting ethanol conversion or cellular growth [45]. On the other hand, laccases and other oxidative enzymes are known to detoxify hydrolysates by removing phenolic compounds such as vanillin, syringaldehyde, ferulic acid and coniferyl aldehyde, which negatively impact the fermentation process of *S. cerevisiae* [47]. Thus, the higher fermentation efficiencies observed in the treatments with enzymes extracts from PlTr co-cultures might be associated with the higher laccase activities (Figure 5) and possibly other oxidative enzymes secreted by Pl.

#### 4. Conclusions

Co-cultures of *T. reesei* RUTC30 ATCC 56765 and *P. lecomtei* BRM 061104 leads to producing extracts with cellulase and high laccase activity, contributing to the best performance of the enzymatic hydrolysis of pretreated sugarcane bagasse. This group of enzymes favors pretreated sugarcane bagasse hydrolysis, producing monomers of great industrial importance, such as glucose and xylose. It was possible to improve the production of enzymatic extracts highlighting the temperature control, time of interval between inoculums and the addition of chemical supplements, such as CuSO<sub>4</sub> or biomass as wheat bran, cottonseed cake or oil palm decanter cake. The hydrolysates obtained here were used by *S. cerevisiae* for ethanol production. These hydrolysates from the co-culture extracts, were shown to be more fermentable than those from monocultures, which could be associated with the presence of oxidative enzymes involved in the detoxification process.

**Author Contributions:** Conceptualization, R.D.R.P., F.G.d.S. and J.R.M.d.A.; Data curation, R.D.R.P., D.W., T.D.M. and T.F.P.; Formal analysis, R.D.R.P., T.D.M. and T.F.P.; Funding acquisition, F.G.d.S. and J.R.M.d.A.; Investigation, R.D.R.P., D.W., J.R.B.C. and F.G.d.S.; Methodology, R.D.R.P., T.D.M. and T.F.P.; Supervision, F.G.d.S. and J.R.M.d.A.; Writing—original draft, R.D.R.P.; Writing—review & editing, F.G.d.S. and J.R.M.d.A. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by Brazilian Agricultural Research Corporation (EMBRAPA) and supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brasília, Brazil.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** University of Brasília (UnB) and Brazilian Agricultural Research Corporation (EMBRAPA).

**Conflicts of Interest:** The authors R.D.R.P., T.D.M., T.F.P. and F.G.S. have a patent application in Brazil (2018) regarding co-culture of fungi for production of lignocellulolytic enzymes.

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