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## Obtaining Enzymatic Extract from *Pleurotus* spp. Associated with an Integrated Process for Conversion of Lignocellulosic Biomass to Bioproducts

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### Abstract

The pretreatment of biomass has been integrated with enzyme production through the recycling of aqueous fractions. A process integrated with *Pleurotus cystidiosus* was grown, and enzymatic hydrolysis was realized. Samples of every liquid fraction from the fungal growing medium were analyzed to determine the chemical oxygen demand (OCD), glucose (Glu), xylose (Xyl), and total reducing sugars (RS). Separately, to obtain valuable polymers from this integration process, solid hemicellulose and lignin were isolated from the remaining liquid fractions through pH variation. The composition of the samples was determined using scanning electron microscopy (SEM), optical stereoscopic microscopy, and Fourier transform infrared (FTIR) spectroscopy and was compared with commercial homologs. The maximum conversion of cellulose to glucose by the obtained liquid fraction of the fungal medium was  $61.3 \pm 0.9\%$  of the theoretical conversion yield of the commercial enzyme. Similarly, the conversion of hemicelluloses to xylose was  $69.5 \pm 1.5\%$ . Finally, in this work, an integrated platform for cellulose, hemicellulose, lignin, enzymatic extract, and sugars production, which also significantly reduces water consumption, was proposed.

**Keywords:** alkaline delignification, cellulose, hemicellulose, lignin, corn straw, *Pleurotus cystidiosus*, biorefinery

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## 1. Introduction

*Pleurotus* spp. is one of most extensively studied white-rot fungi for its exceptional ligninolytic properties [1]. This genus cleavages cellulose, hemicellulose, and lignin from wood, whereas brown rot fungi only cleavage cellulose and hemicellulose [2]. In basidiomycete fungi, extra-cellular laccases are constitutively produced in small amounts, and the lignocellulolytic enzymes are affected by many typical fermentation factors, such as medium composition, pH, temperature, aeration rate, etc. [3–5]. Mushroom survival and multiplication are related to a number of factors: chemical composition, water activity, ratio of carbon to nitrogen, minerals, surfactant, pH, moisture, sources of nitrogen, particle size, amount of inoculum, antimicrobial agents, and the presence of interactions between microorganisms. *Pleurotus* spp. is a saprophyte, and it extracts its nutrients from the substrate (grasses, wood, and agricultural residues) through its mycelium, obtaining substances necessary for its development, such as carbon, nitrogen, vitamins, and minerals. Agro-industrial waste is produced in huge amounts, and it becomes an interesting substrate for basidiomycete fungi. Many studies have been conducted to test the ability of *Pleurotus* spp. to grow on different agro-wastes, such as rice straw, wheat straw, and corn straw [6, 7].

Mexico is the third largest country in LAC in terms of the cropland area and would become a central focus of attention to produce bioproducts. It was estimated that 75.73 million tons of dry matter was generated from 20 crops in Mexico. From this biomass, 60.13 million tons corresponds to primary crop residues mainly from corn straw, sorghum straw, and wheat straw. The generation of secondary crop residues accounted for 15.60 million tons to which corncob was one the main contributors. Corn straw is the first most abundant crop residue generated in Mexico, equivalent to 66.9% of the total amount of crop residues from the cereal agro-industry [8]. The amount of corn straw reached a total of  $25.1 \times 10^6$  tons, and the State of Guanajuato is the seventh largest national corn straw producer with almost  $1.3 \times 10^6$  tons per year, equivalent to 5.3% of the gross production [9, 10]. This residue is mostly left to decompose or burn in situ, generating serious problems of atmospheric emissions. Nevertheless, a change in the agro-industry research topics in Mexico is emerging, where novel sustainable solutions for solid waste management are being pursued. Mexico's economic growth prospects have emphasized the importance of the development of clean technologies, in which lignocellulosic biomass conversion processes are integrated with minimum residue generation for the sustainable production of biofuels and bioproducts [11]. Three general steps are involved when transforming a lignocellulosic residue into a value-added by-product: (1) biomass pretreatment, (2) hydrolysis of the polysaccharides, and (3) fermentation of sugars. Chemical, thermochemical, and biological treatments are intended to (1) increase the formation of fermentable sugars or increase the ability to form sugars in a subsequent enzymatic saccharification step by strategically breaking the highly ordered carbohydrate structure to improve enzyme access, (2) remove or partially depolymerize lignin, (3) avoid the formation of products that are saccharification or fermentation inhibitors, and, finally, (4) be economically feasible. The alkaline/hydrogen peroxide pretreatment of corn straw (CS), also known as alkaline oxidative (AlkOx) delignification, causes the separation of biomass into its principal components: cellulose, hemicelluloses, and lignin. It also presents minimum carbohydrate

degradation while enhancing further enzymatic saccharification, favoring economic feasibility. The AlkOx delignification generates products that are not limited to monosaccharides or bioethanol. They can also be commercialized as feedstocks for making composites, biodegradable packaging materials, construction materials, paper and board, easily digested cattle food, and substrates for mushroom cultivation, among other products. Alkaline pretreatment can be performed at room temperature and times ranging from seconds to days [12]. Therefore, it can reduce energy costs. Pretreatment is the key to unlocking low-cost cellulosic biomass; the pretreatment methods ought work on a widespread spectrum of feedstocks, have minimum preparatory processes, and provide a cellulosic current that can be efficiently hydrolyzed with low concentrations of enzyme [13]. In comparison with acid processing, alkaline processing features less sugar degradation and recovery or regeneration of many of the caustic salts. This leads to the reduction of costs in the process. The treatment of the water and treatment of the residues are two challenging stages for biorefineries. In this sense, as the alkaline hydrogen peroxide pretreatment of corn straw (CS) separates the biomass into cellulose, hemicelluloses, and lignin in different percentages, it enhances enzymatic cellulose hydrolysis and minimizes cellulose loss. Its products are not limited to C5/C6 sugars but can also be commercialized for polymeric applications [14, 15]. On-site production of biomass-degrading enzymes can assist in achieving full supply independence. The interest in finding new alternative treatments, such as the utilization of white-rot fungi to degrade lignocelluloses, is increasing. The white-rot fungus *Pleurotus* spp. has the ability to degrade and metabolize lignin as well as other sugars. *Pleurotus cystidiosus* is a fungus with immense biodegradation potential by the effect of its enzymes lignin oxidase, lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase on the degradation of lignocellulose of corn stover. Laccases or ligninolytic peroxidases (LiP and MnP) oxidize the lignin polymer, thereby generating aromatic radicals. These evolve in different nonenzymatic reactions, including C4-ether breakdown, aromatic ring cleavage, C $\alpha$ -C $\beta$  breakdown, and demethylation. The aromatic aldehyde releases from C $\alpha$ -C $\beta$  breakdown of lignin or synthesized by fungi are the substrates for H<sub>2</sub>O<sub>2</sub> generation by aryl-alcohol oxidase in cyclic redox reactions involving also aryl-alcohol dehydrogenases. Phenoxy radicals from C4-ether breakdown can depolymerize on the lignin polymer if they are not first reduced by oxidases to phenolic compounds. Then, lignin degradation proceeds by oxidative attack of the enzymes [16]. The objective of this work was to isolate and characterize cellulose, hemicellulose, and lignin from corn straw and the sugar concentration obtained from hydrolysis by the enzymatic cocktail from *P. cystidiosus* in an integrated scheme.

## 2. Materials and methods

Corn straw (*Zea mays*) was harvested at a local farm near the city of Manuel Doblado (location within the state of Guanajuato, Mexico; coordinates 20°45'08.30"N 101°56'07.31"O). We were unable to identify the crop genotype, but the most common variety used in the region is "H 368 C (1313-MAZ-556-010900/C)" developed by INIFAP (National Institute of Forest, Agricultural and Livestock Research). The planting was carried out in the month of January 2016 using common methods of 1.5 m between rows, 120 kg of seed per ha, and three irrigations in total and fertilized with phosphorus (30%) and urea with an average content of 46% nitrogen. The



at 450 rpm. Then,  $35 \pm 5$  mL of 50% (w/v) (10 M) NaOH (J.T. Baker ACS Reagent Grade) solution was added until a pH of  $11.5 \pm 0.3$  was reached. After that, 40 mL of 50% (w/v)  $H_2O_2$  (J.T. Baker ACS Reagent Grade) was added, and the pH was again adjusted to 11.5 through the dropwise titration with  $4.5 \pm 1.5$  mL of 5 M HCl (J.T. Baker ACS Reagent Grade) solution, giving an approximate dilution rate of 6% (w/v) for the CS. The suspension was then stirred gently for 5 h.

## 2.2. Isolation of bioproducts

After treatment, solid and liquid fractions were separated by filtration. The solid fraction, or pretreated corn straw (PCS), was washed five times with distilled water (EcoPura SA De CV, Santiago de Queretaro, Qro., Mexico) until the pH of the filtrate was neutral, consuming 150–250 mL of water per gram of straw. Then, PCS was dried at  $50^\circ\text{C}$  for 24 h. The solid fractions after AlkOx treatment were mainly formed of crude cellulose. The water was recovered (RWW) and reintegrated in subsequent processes, as depicted in **Figure 1**. The liquid fraction (FCS) was kept and stored at  $4^\circ\text{C}$ , until further use. Hemicellulose was obtained by precipitation. First, the FCS was adjusted to pH 5.5 with 5 M HCl. The solubilized hemicelluloses were precipitated by adding anhydrous ethanol at a 3:1 (v/v) ratio. Then, the sample was centrifuged at 7000 rpm for 10 min, and, finally, the solid was dried in a convection oven at  $60^\circ\text{C}$  for 8 h. Lignin was obtained by drying the remaining liquid fraction for 6 days at  $25^\circ\text{C}$ .

## 2.3. Characterization

Infrared spectrum of PCS, hemicellulose, and lignin were obtained with a PerkinElmer Spectrum One FTIR spectrophotometer (Perkin Elmer de Mexico, SA, Mexico, DF) using the attenuated total reflection (ATR) device. Thirty-two scans were taken from each sample and recorded from  $4000$  to  $650\text{ cm}^{-1}$  at a resolution of  $2\text{ cm}^{-1}$  in transmission mode. Automatic ATR, baseline correction, and normalization with a factor of 0.5 were performed on each spectrum. For the scanning electron microscopy (SEM) imaging, the untreated (UCS) and PCS were sputter coated with gold for 100 s. The coated samples were observed using a JEOL JSM-35CF microscope (JEOL de Mexico SA De CV, Mexico, DF) operated at 15 kV. Stereoscopic images were taken with an optical stereoscopic microscope (OSM); the model was Euromex Novex trinocular zoom stereo microscope, model RZT-SF 65.560 (Westek, SA DE CV, Guadalajara Jal., Mexico).

## 2.4. Submerged culture of mycelium

The white-rot fungus was isolated from local wood residues, which was identified as *Pleurotus cystidiosus* strain P-24 (GenBank FJ379283.1) by the Instituto Potosino de Investigacion Científica y Tecnológica (IPICYT, San Luis Potosí, SLP, Mexico). The fungus was cultured in 125 mL Erlenmeyer flasks containing 50 mL of FCS or recycled FCS (RFCS) at pH 5.5 after inoculation with two 2 mm spheres sampled from a plate with PDA on which the fungal mycelia had been cultured. The flasks were set in an orbital shaker incubator at  $28^\circ\text{C}$  and 100 rpm for 5 days. Then, filtrates were obtained (MycFCS and MycRFCS) from the cultures.

## 2.5. Enzymatic hydrolysis

The enzyme Accellerase 1500™ for the saccharification step was provided by DuPont™ Genencor® Science (DuPont, Wilmington, Delaware, USA) [18]. The filter paper activity was determined by the standard procedures recommended by the National Renewable Energy Laboratory (NREL/TP-510-42,628). The hydrolysis of the PCS was performed in a thermo-mixer (Eppendorf Thermomixer Comfort, Surtidor Quimico del Centro SA De CV, Santiago de Queretaro Qro., Mexico) at 40°C and 100 rpm [19]. The samples were placed in 1.5 mL microcentrifuge tubes containing 1% w/v of dry PCS with either distilled water or MycFCS or MycRFCS cultures. The experiments were run as follows: PCS + MycFCS, PCS + MycRFCS, PCS + MycFCS + Accellerase, PCS + MycRFCS + Accellerase, and PCS + Accellerase. Each experiment was stopped at different times (24, 48, and 120 h), alone or mixed with an enzyme loading of 16 FPU g<sup>-1</sup> of PCS at pH of 5.0 (0.1 mol L<sup>-1</sup> sodium acetate buffer), with sodium azide (NaN<sub>3</sub>, 0.01% (w/v)) to prevent microbial growth.

## 2.6. Analysis of the liquid phases

Hydrolysate was separated from the solid residue by centrifugation at 8608.6 g-force (Eppendorf rotor FA-45-18-11, Equipar SA De CV, Ciudad de Mexico, Mexico) for 10 min. The total reducing sugars (RS) were measured using the Miller method [20]. The measurement of glucose (Glu) and xylose (Xyl) was accomplished in the biochemical analyzer 2700 SELECT from YSI (YSI Life Sciences, Yellow Springs, Ohio, USA) using the Xylose Software. The OCD analyses were run according to the standard method for the analysis of potable and residual water [21]. All these experiments were conducted in triplicate.

## 2.7. Data analysis

Statistical analysis was developed in order to determine the statistical differences between treatments against time using the software SAS JMP version 13.1.0 (SAS Inc., Cary, North Carolina). First, a one-way analysis of variance (ANOVA) was carried out to determine if the results obtained using different treatments were significantly different. Then, if the ANOVA confirmed the existence of a significant difference ( $p < 0.05$ ), a post hoc analysis (Tukey's HSD) was used to determine between which values the difference was significant, considering a level of significance of 0.05.

## 3. Results and discussion

The samples of CS had the following composition (% w/w): 43.5% cellulose, 35.8% hemicellulose, 11.1% lignin, and 9.2% other components on a dry weight basis (**Table 1**). The lignin content of CS was slightly lower than in other reported works [22–24]. However, within the range, it coincides with other published works [25, 26]. Considerable variation in feedstock composition may occur because the corn straw is grown in different environments. In addition, there can be genotypic differences. Feedstock composition varies with crop maturity.

| Components    | Percentage (%) |
|---------------|----------------|
| Dry matter    | 90.2 ± 3.3     |
| Cellulose     | 43.5 ± 1.6     |
| Hemicellulose | 35.8 ± 1.2     |
| Lignin        | 11.1 ± 1.8     |
| Ash           | 9.2 ± 2.0      |
| Calcium       | 0.3 ± 0.1      |
| Phosphorus    | 0.05 ± 0.04    |

Bromatological analysis of WS realized by the University Center for Biological and Agricultural Sciences (CUCBA) at the University of Guadalajara, Mexico, using the official methods of National Renewable Energy Laboratory (NREL).

**Table 1.** Chemical components of corn straw (CS) from Manuel Doblado, Gto., Mexico (20°43'42"N 101°56'57"O).

Maximum lignocellulosic yield is obtained when corn is harvested at physiological maturity. When the corn straw is harvested before and after physiological maturity, the compositions of lignocellulose are found to be distinct.

### 3.1. AlkOx delignification

*Cellulose, hemicellulose, and lignin contents* after the pretreatment are presented in **Table 2**. Compositional analysis of the PCS showed that it had 75.1% cellulose, 5.9% hemicelluloses, and 0.6% lignin. Under these conditions, 83.5% of the hemicellulose and 93.4% of the lignin were removed after pretreatment. Previous reports about the alkaline oxidative pretreatment of corn straw for bioproduct production are similar [26–30]. Comparing the results with other studies for the delignification of CS, the lignin content was slightly lower than that obtained by us [27]. Gould [31] demonstrated the use of H<sub>2</sub>O<sub>2</sub> for delignification with a maximum pH of 11.5. The porosity of the lignocellulosic materials increases with the removal of the cross-link dilute NaOH treatment of lignocellulosic materials causing swelling, leading to an increase in internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, a separation of structural linkages between lignin and carbohydrates, and a disruption of the lignin structure [32]. On the other hand, the pretreatment of CS with hydrogen peroxide enhanced its susceptibility to enzymatic hydrolysis. As seen in **Table 2**, Xyl was the most abundant sugar present, suggesting that some hemicellulose degradation had occurred. This is due to the partial hemicellulose degradation, and its magnitude increases proportionally with the temperature, pH, and reaction time period. On the other hand, during this process, the reuse of wastewater from washing of the PCS resulted in low water consumption (**Figure 1**). The quantity of water used to wash was about 1.5 L per 50 g of the PCS (it should be carried out at pH 6.0 ± 1.5), which was reintegrated to 100% into the process. Considering the abovementioned, 30,000 L of water could be wasted per 1 ton of the PCS into the process. It is noteworthy that the moisture content of PCS was between 8 and 12%. This AlkOx pretreatment, as proposed in this study, has several potential advantages over the alkaline wash step, because it presents a higher amount of sugars, higher solid loadings can be treated, and

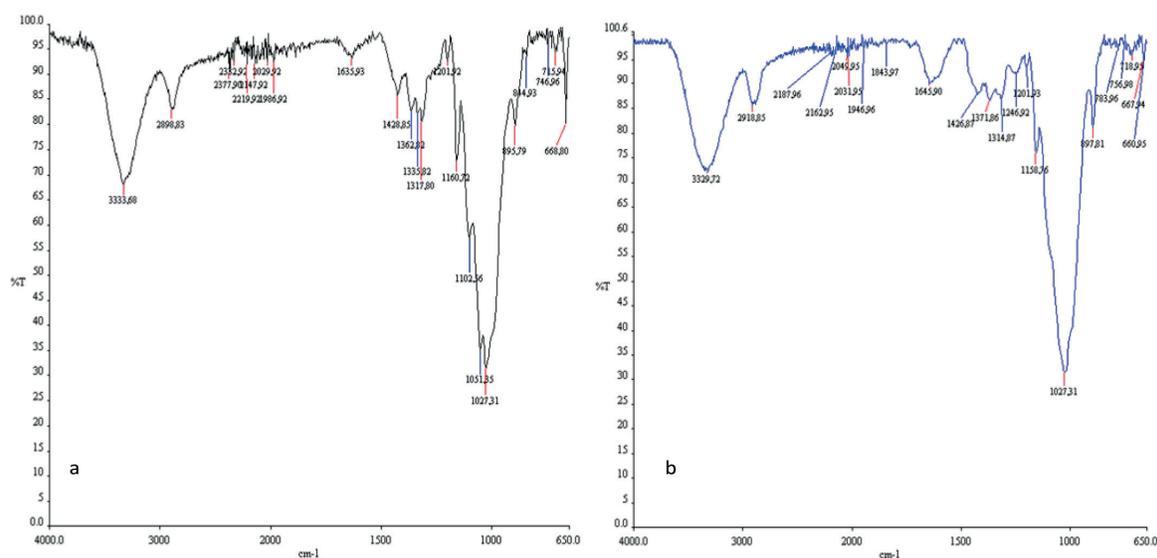
| Pretreated wheat straw (PWS) | Cellulose (%) | Hemicellulose (%) | Lignin (%) | RS (%)     | Glu (%)    | Xyl (%)    |
|------------------------------|---------------|-------------------|------------|------------|------------|------------|
| 6 mm screen size WS          | 75.1 ± 2.1    | 5.9 ± 1.1         | 0.6 ± 0.1  | 0.7 ± 0.08 | 0.2 ± 0.02 | 0.4 ± 0.05 |
| Component removed (CR)       | —             | 83 ± 1.1          | 93 ± 1.3   | —          | —          | —          |
| Kristensen [58]              | 75            | 90                | 100        | —          | —          | —          |
| Asghar [29]                  | 83            | 90                | 81         | —          | —          | —          |

**Table 2.** Compositional analysis of the pretreated corn straw (solid fraction), total reducing sugars (RS), glucose (Glu), and xylose (Xyl) released (liquid fraction) and references for component removal.

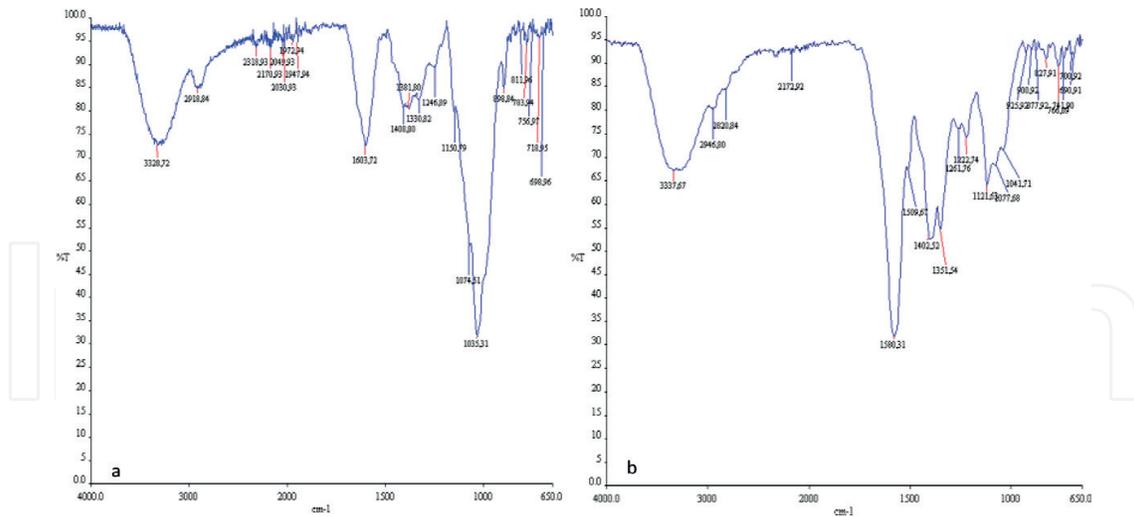
a significantly lower amount of water is required. All of these aspects have a direct impact on the manufacturing costs and energy requirements of the process [33].

### 3.2. Characterization

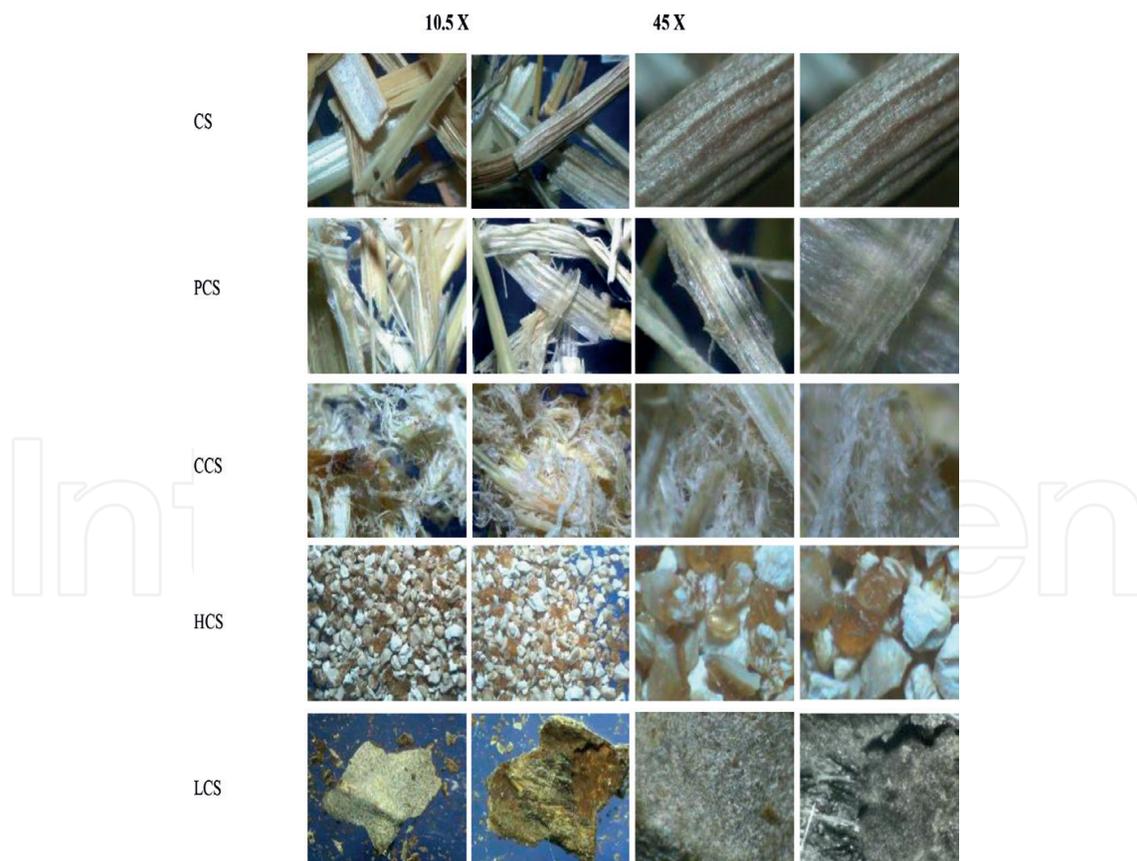
Results from FTIR spectroscopy, SEM, and OSM were used to compare bioproducts (cellulose, hemicellulose, and lignin) obtained, as well as information on the effect of the pretreatment on the structure and possible disruption of the cell wall (Figures 2–5). The FTIR spectrum of the commercial cellulose and the PCS were similar (Figure 2a and b). The peaks at 1165 at 1059  $\text{cm}^{-1}$  were assigned to the linkage present in the cellulose and can be associated to  $\beta(1-3)$ -polysaccharide, which is a strong signal characterizing a high cellulose composition [34]. The transmittance at 1429, 1363, 1317, 1161, 1051, and 896  $\text{cm}^{-1}$  (Figure 2a and b) is a typical pure cellulose that are associated with  $\text{CH}_2$  in-plane bending vibrations, with  $\text{C}=\text{O}$  stretching, and with  $\text{C}-\text{H}$  ring in-plane bending vibrations [35–37]. The absence of a band



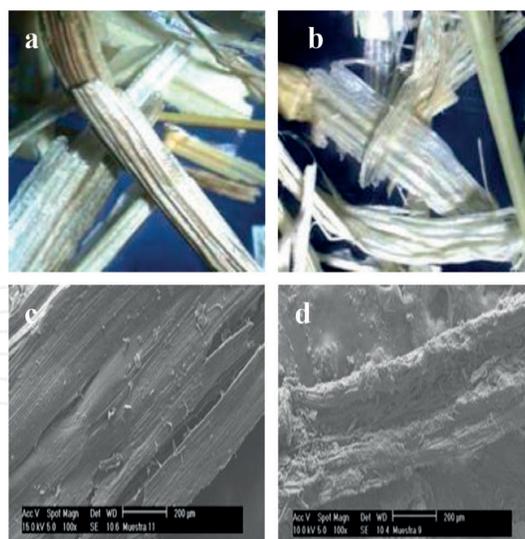
**Figure 2.** Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectra of (a) commercial cellulose and (b) pretreated corn straw.



**Figure 3.** Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectra of (a) hemicellulose and (b) lignin from alkali pretreatment of corn straw.



**Figure 4.** Images obtained by optical stereoscopic microscopy of the untreated (CS), pretreated (PCS), cellulose (CCS), hemicellulose (HCS), and lignin (LCS) from corn straw.



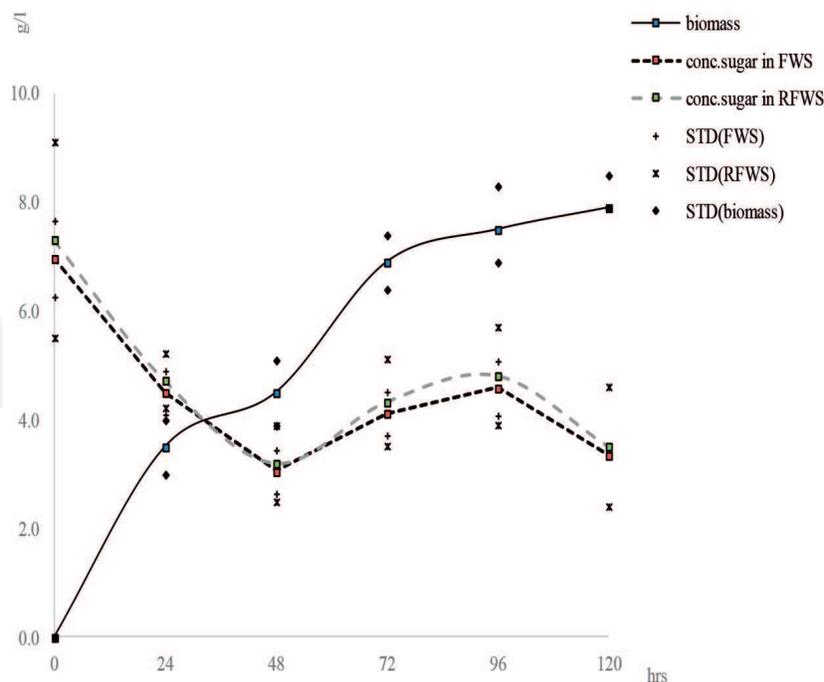
**Figure 5.** Optical stereoscopic microscopy (a, b at 10.5 $\times$ ) and scanning electron microscopy (c, d at 100 $\times$ ) images of the untreated (a, c), and pretreated corn straw (b, d).

at 1731  $\text{cm}^{-1}$  indicates cleavage of the acetyl and uronic ester groups from the hemicelluloses, while the absence of the absorption band at 1750–1700  $\text{cm}^{-1}$  region and the peak relating to lignin aromatic ring vibrations at 1513  $\text{cm}^{-1}$  revealed that lignin was removed during the alkaline peroxide process [34]. These results corroborate the ones obtained for the percentage composition of the PCS obtained in the present work. **Figure 3** shows the FTIR spectrum of the hemicelluloses and lignin. The absorbance at 2919, 1408, 1382, 1247, 1075, 1036, 985, and 899  $\text{cm}^{-1}$  is associated with hemicelluloses, in which 1039  $\text{cm}^{-1}$  is typical of arabinoxylans (**Figure 3a**). The FTIR spectrum of lignin is shown in **Figure 3b**. The C—H stretching vibration gives signals at 2919–2820  $\text{cm}^{-1}$  [38]. It is noteworthy that the structure of lignin varies with different types of agricultural crops [39, 40]. In the case of the spectrum, an intensive band at 1580  $\text{cm}^{-1}$  was observed, while it was a weak peak at 1509  $\text{cm}^{-1}$ , which are associated with quadrant ring stretching and semicircle ring stretching (aromatic lignin) [34]. In addition, the absorption at 3334–3328  $\text{cm}^{-1}$  is attributed to the stretching of —OH groups that may include absorbed water [27]. The C—H stretching vibration gives signals at 2919–2820  $\text{cm}^{-1}$ . The band at 1646–1636  $\text{cm}^{-1}$  is due to the bending mode of absorbed water [27]. Specific band in the 1200–1000  $\text{cm}^{-1}$  regions is dominated by ring vibrations overlapped with stretching vibrations of side groups (C—OH) and the glycosidic bond vibration (C—O—C) [34]. The high absorbance at 1351  $\text{cm}^{-1}$  arises from the C—C and C—O skeletal vibrations [34]. **Figure 4** shows the different structures of the products obtained from the corn straw by OSM. PC is clearly observed that color changes from brown to slightly white, as well as structural deformation of fiber cells and particle fragmentation by pretreatment, indicate lignin removal. In the cellulose image, the fibrous structure is observed, unlike the hemicellulose. Hemicellulose shows a white to yellow and faint brown to brown color. Other works have mentioned that, at high NaOH loadings (above 260 mg NaOH/g dry straw), there is a diminishing effect on lignin removal as the NaOH loading is increased [41], so that the pretreated solids range from dark brown to white as more lignin is removed.

**Figure 5** shows the characterization of PCS and UCS by SEM and OSM. Lignin shows a light brown color, and it presents a rubberlike appearance. These characteristics are in accordance with the standards described by commercial houses [42]. As seen in **Figure 5a** and **c**, the OSM image and SEM micrograph, the anatomy of the UCS and PCS is easily recognizable, the thick-walled fiber of the straw wall can be seen, as well as it is largely made of wax. The most evident effect of the pretreatment is the separation of individual fibers of the CS. On the other hand, the pretreated material is quite heterogeneous and contains pieces of different sizes (<1 cm) (**Figure 5b**). Cracks and holes were seen in the structure of the corn straw (**Figure 5d**). This shows that it is easily digestible by enzymes [16, 17].

### 3.3. Submerged culture of the mycelium

**Figure 6** shows the time course of the biomass of the mushroom strain (*P. cystidiosus*), and the sugar concentration is reported for the onset of mushroom growth. The RS concentrations from FCS and RFC were  $6.9 \pm 0.7$  and  $7.3 \pm 1.8$  g/L. Percentage contents of xylose were 68.1 and 73.4%, respectively. This bioprocess presented an interesting behavior. A diauxic growth was observed, an inflection in the growth curve or even a decline in biomass occurs at 48 h. Under these conditions, 0.45% (w/v) of the amount of biomass was increased to 0.69% (w/v) at 72 h, which represents 53% (w/w). The presence of a preferential carbon substrate may inhibit the synthesis of the enzymatic system involved in the uptake and metabolism of a second carbon substrate. The *P. cystidiosus* culture may metabolize xylose preferentially in a mixed substrate medium containing xylose and other sugars. The product of xylose metabolism would be

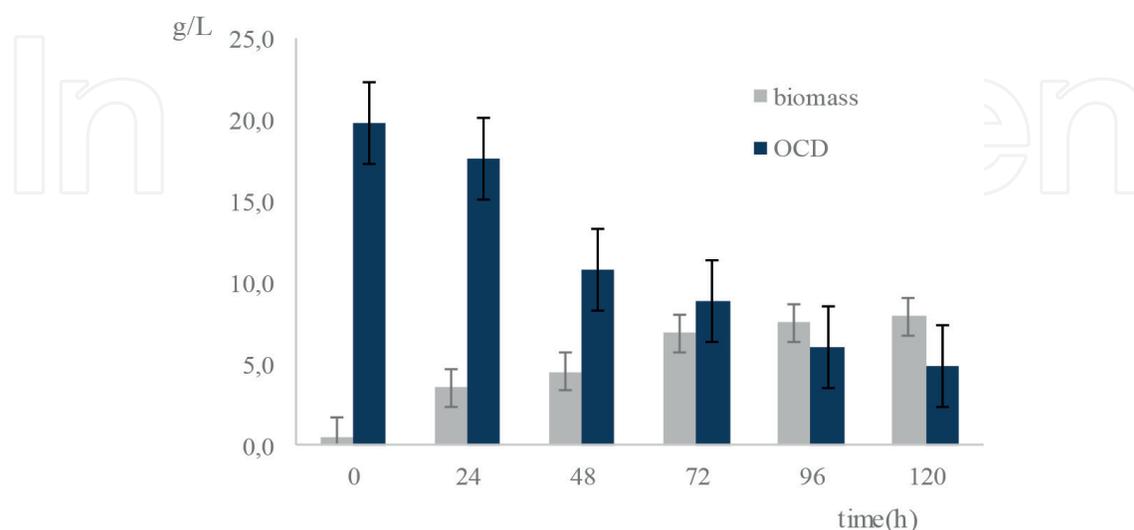


**Figure 6.** Growth kinetic of *Pleurotus cystidiosus* mycelium in culture media based on the filtrate from the process of PCS (FCS and RFC). Standard deviation (STD).

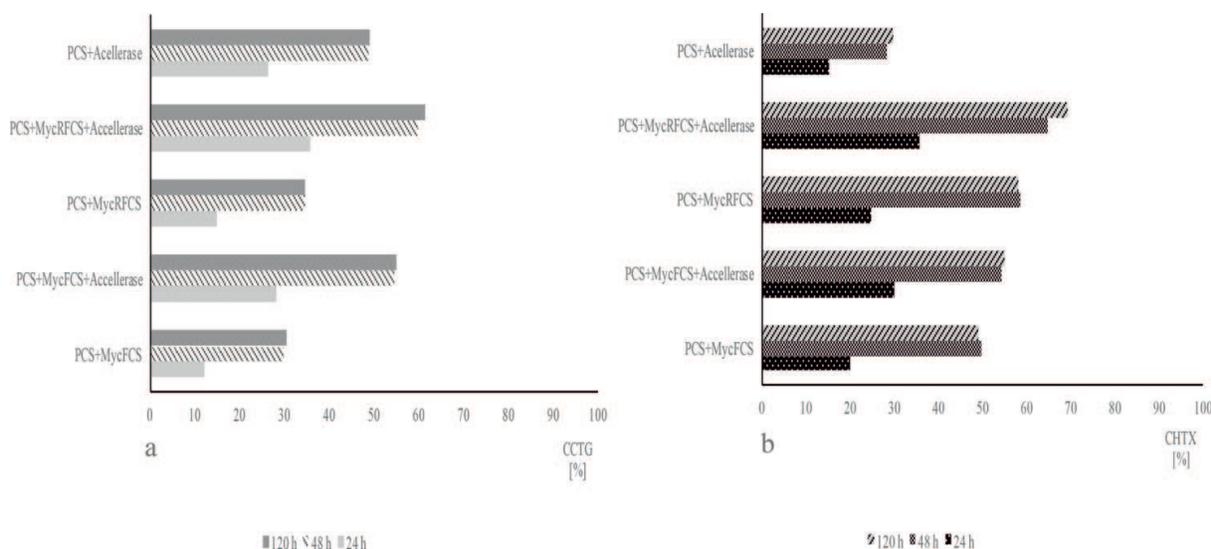
utilized as a carbon source only when the principal one is exhausted. There are few reported works on the growth of *P. cystidiosus*; these mention fungi grown on a medium containing glucose [43, 44]. However, xylose is shown as a good carbon source by several works [45]. The RS concentration after 96 h decreased to 73%, coinciding with the poor growth of biomass. In this context, **Figure 7** shows the relation between OCD and growth of biomass. The OCD was reduced to 75%, which is congruent with the increase in biomass. In the stationary phase (72 h), oxygen consumption dropped drastically, indicating a decrease in cellular aerobic metabolism, which may be associated with lack of nutrients in the medium or loss of cellular viability. In the same context, in spite of the numerous potential applications of the *Pleurotus* spp. enzymatic system, little is known about the production of enzymes, in which dissolved oxygen concentration, among other parameters, can be strictly controlled [46].

### 3.4. Enzymatic hydrolysis

**Figure 8a** shows the conversion of cellulose to glucose due to hydrolysis using MycFCS and MycRFCS, alone or mixed with Accellerase 1500. **Figure 8b** shows the conversion of hemicellulose to xylose. The maximum sugar concentration was obtained by PCS + MycRFCS + Accellerase for 120 h, with 6.8 g/L of RS, which included 4.6 g/L Glu and 0.41 g/L Xyl, representing a conversion of cellulose to glucose (CCTG) about 61.3% and a conversion of hemicellulose to xylose (CHTX) around 69.5%. When only the MycFCS medium was used, the optimal result occurred when using PCS + MycRFCS-48 h with a conversion and a net RS increment of 2.9 g/L, which included 2.6 g/L Glu and 0.30 g/L Xyl, representing a CCTG of 34.7% and CHTX of 50.8%. In the case of PCS + MycFCS, the results obtained for CCTG at 48 and at 120 h were 29.7 and 30.5%, respectively. Within the same case, for CHTX were 49.9 and 49.1%. The lowest concentrations were for the experiment PCS + MycFCS for 24 h, 12.2% of CCTG, and 20.1% of CHTX. In all cases, adding Accellerase resulted in slightly higher (CCTG (28.1–61.3%) and CHTX (30–69.5%)); however, the concentrations were lower when Accellerase was used without any MycFCS or MycRFCS (CCTG (26.3–49.1%) and CHTX (15.2–29.7%)). Therefore, a synergistic effect was



**Figure 7.** The relation between chemical oxygen demand and growth of biomass.



**Figure 8.** (a) Conversion of cellulose to glucose due to hydrolysis using the filtrates from the growth of the fungus (MycFCS and MycRFCS), alone or mixed with Accellerase 1500. (b) Conversion of hemicellulose to xylose due to hydrolysis using the filtrates from the growth of the fungus (MycFCS and MycRFCS), alone or mixed with Accellerase 1500.

observed among the mixtures. Some studies have reported the conversion of cellulose to glucose above 80%; in our case the results were lower than expected [47, 48]. This might be due to the amounts of the substrate released to the MycFCS or MycRFCS, inhibiting the enzymatic hydrolysis process. On the other hand, it is documented that all the species of *Pleurotus* produced laccase, manganese peroxidase, and aryl-alcohol oxidase activity [49–51]. Then, this may have an effect of competitive inhibition with enzyme complex from Accellerase, which contains multiple enzyme activities: exoglucanase, endoglucanase, hemicellulase, and beta-glucosidase. However, one of the important contributions of this work is that *P. cystidiosus* cultured in lignocellulosic substrates in FCS or RFCS was found to secrete a range of important degradative enzymes, such as xylanase (CHTX (20.1–50.8%)). Moreover, it should be noted that the MycFCS or MycRFCS comes from the mycelial stage of the fungus. Otherwise, Ellisahvilli observed that laccase activity of *Pleurotus* was high during the colonization stage and declined during the first primordial formation and fruiting stage [52]. Similar result was reported by Malarczyk in *P. cystidiosus* where laccase was active during mycelial growth on solid saw dust [53]. The enzymes associated with lignin-degrading ability of white-rot fungi are lignin peroxidase, manganese peroxidase, laccase, and xylanases [54]. Although it is known that white-rot fungi are known to employ a variety of extracellular ligninolytic enzymes, in the case of the *P. cystidiosus*, there are few reported works, and little attention has been given to the evaluation of the hydrolytic system of this fungus [55–58].

### 3.5. Data analysis

The concentration range of RS was 1.33–6.9 g/L. The arithmetic average and relative standard deviation of all analyses are performed; sample size in  $n = 45$  was  $4.5 \pm 1.7$  g/L. Similar results are found in treatments PCS + MycFCS (Treatment 1) and PCS + MycRFCS (Treatment 3),

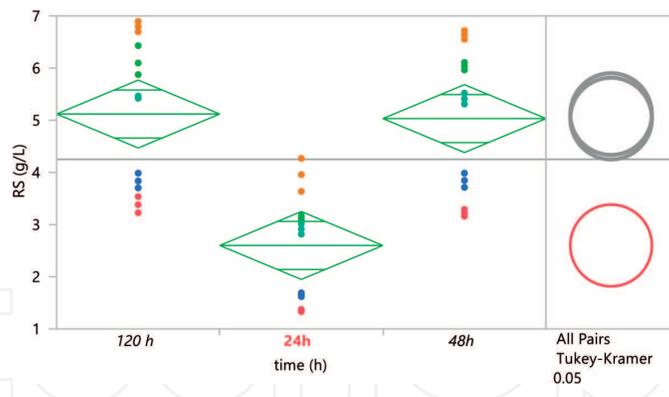


Figure 9. Univariate analysis and comparison means (Tukey’s HSD) for RS from PCS by the effect of enzymatic hydrolysis time.

| Source    | Degrees of freedom | Sum of squares | Mean square | F ratio | Prob > F |
|-----------|--------------------|----------------|-------------|---------|----------|
| Treatment | 4                  | 63.6           | 15.9        | 10.1    | <.0001   |
| Time (h)  | 2                  | 61.356         | 30.678      | 19.6472 | <.0001   |

Table 3. Analysis of variance for the statistical differences between RS from PCS by different enzymatic hydrolysis treatments and time (h) using the software SAS JMP version 13.1.0 (SAS Inc., North Carolina).

| Level                   | - Level                | Difference | Std Err Dif | Lower | Upper | p-Value |  |
|-------------------------|------------------------|------------|-------------|-------|-------|---------|--|
| PCS+MycRFCS+Accellerase | PCS+MycFCS             | 3.14       | 0.59        | 1.45  | 4.84  | <.0001  |  |
| PCS+MycRFCS+Accellerase | PCS+MycRFCS            | 2.68       | 0.59        | 0.99  | 4.38  | 0.00    |  |
| PCS+MycFCS+Accellerase  | PCS+MycFCS             | 2.45       | 0.59        | 0.75  | 4.14  | 0.00    |  |
| PCS+MycFCS+Accellerase  | PCS+MycRFCS            | 1.98       | 0.59        | 0.29  | 3.68  | 0.01    |  |
| PCS+Accellerase         | PCS+MycFCS             | 1.94       | 0.59        | 0.24  | 3.63  | 0.02    |  |
| PCS+Accellerase         | PCS+MycRFCS            | 1.48       | 0.59        | -0.22 | 3.17  | 0.11    |  |
| PCS+MycRFCS+Accellerase | PCS+Accellerase        | 1.20       | 0.59        | -0.49 | 2.90  | 0.27    |  |
| PCS+MycRFCS+Accellerase | PCS+MycFCS+Accellerase | 0.70       | 0.59        | -1.00 | 2.39  | 0.76    |  |
| PCS+MycFCS+Accellerase  | PCS+Accellerase        | 0.51       | 0.59        | -1.19 | 2.20  | 0.91    |  |
| PCS+MycRFCS             | PCS+MycFCS             | 0.46       | 0.59        | -1.23 | 2.16  | 0.94    |  |
| 120 h                   | 24h                    | 2.52       | 0.46        | 1.41  | 3.63  | <.0001  |  |
| 48h                     | 24h                    | 2.43       | 0.46        | 1.32  | 3.54  | <.0001  |  |
| 120 h                   | 48h                    | 0.09       | 0.46        | -1.02 | 1.20  | 0.98    |  |

Table 4. Ordered difference report between RS from PCS by different enzymatic hydrolysis treatments and time (h).

PCS + MycFCS + Accellerase (Treatment 2), and PCS + MycRFCS + Accellerase (Treatment 4). The ANOVA applied to PCS + Accellerase (Treatment 5) shows that significant statistical differences (SSD) exist between all treatments and are possible to suggest that the experiment could be performed over a long time with respect to orders. **Figure 9** shows that results were very similar to each other where there are no SSD between the results obtained at 48 and 120 h, but all the treatments showed SSD with respect to 24 h. The results suggest that the process is completed at 48 h. **Tables 3** and **4** show the analysis of variance for the statistical differences between RS from PCS by different enzymatic hydrolysis treatments and time (h), and the ordered differences report hydrolysis treatments and time (h), respectively.

## 4. Conclusions

The integrated scheme with the use of the filtrate from the pretreatment of the CS and the growth conditions of *P. cystidiosus* studies showed a great potential for the production of lignocellolytic enzymes and application of crude enzymatic extract by the fungus. Replacing commercial enzymes with locally produced enzymes may reduce the cost of enzymatic saccharification, (e.g., the approximate cost of commercial cellulose from Sigma-Aldrich is about \$110 USD of 50 mL of aqueous solution ( $\geq 700$  units/g)), which may translate to high-impact savings in the production cost of fermentable sugars. From a perspective of clean and sustainable technologies, a very important contribution of this work is the reuse of effluents from the treatment of the delignification of lignocellulosic waste to integrate them within the same chain of processes. This way to reduce considerably the use of resources like water and raw material in parallel reduces cost of production. In addition, the utilization of agro-industrial wastes as feedstock in the production of enzymes is more economical and profitable. The high cost of raw materials in the production of enzymes is around 40-60%. Therefore, agro-industrial wastes strategically represent low-cost raw materials, due to their high biodegradability and rich in carbon, to be used as substrates in the chain of production of enzymes. Another important contribution is about the fate of the corn straw. One of the environmental problems in several regions of Mexico is because the straw is burned by the farmers. Therefore, using it as a raw material for obtaining high-value-added bioproducts contributes to improve air quality, environment, and quality of life. Finally, these results can be part and contribute to the development of a biorefinery which can be applied in the treatment of lignocellulosic waste. Undoubtedly, in Mexico, the biorefineries represent great opportunities to harness the economic benefit of agro-industrial waste and to develop even more efficient and sustainable systems.

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## List of abbreviations

|         |  |
|---------|--|
| CS      | corn straw                                 |
| UCS     | untreated corn straw                       |
| PCS     | pretreated corn straw                      |
| FCS     | liquid fractions from PCS                  |
| RFCS    | recycled FCS                               |
| MycFCS  | filtrates of cultures from FCS + mycelium  |
| MycRFCS | filtrates of cultures from RFCS + mycelium |
| SSD     | significant statistical difference         |
| STD     | standard deviation                         |

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