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A STRATEGY FOR COMBINED ENZYME AND BIOGAS PRODUCTION FROM WHEAT STRAW IN A BIOREFINERY

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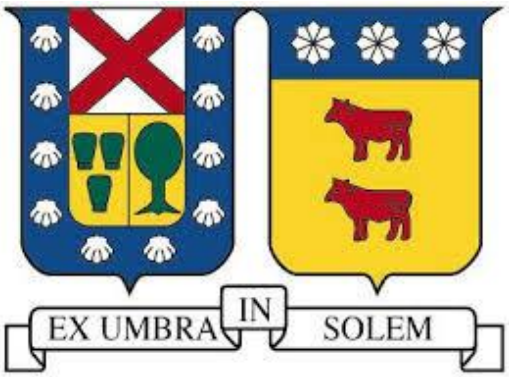

UNIVERSIDAD TÉCNICA FEDERICO SANTA MARÍA

DEPARTAMENTO DE INGENIERÍA QUÍMICA Y AMBIENTAL

A STRATEGY FOR COMBINED ENZYME AND BIOGAS PRODUCTION FROM WHEAT STRAW IN A BIOREFINERY

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Santiago, Julio 2017

To my family and friends…

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RESUMEN

Debido a diversos problemas medioambientales, se necesita maximizar el valor económico de los residuos lignocelulósicos (LCW) dentro de un concepto de biorefinería. La digestión anaeróbia (AD) puede usar los LCW para producir biogás. Además, la aplicación de un tratamiento fúngico antes de la AD puede mejorar el proceso y producir enzimas de alto valor como lacasa (Lac) y manganeso peroxidasa (MnP). Sin embargo, tiempos de tratamiento largos y alta pérdida de carbohidratos, son algunas de las variables que afectan la digestión anaerobia en gran medida.

Para el tratamiento fúngico, se utilizaron los hongos de pudrición blanca *Trametes versicolor, Pleurotus eryngii* y *Pleurotus ostreatus*, sobre paja de trigo previamente descontaminada en dos tamaños de partícula (1.4 a 2.36 mm y 0.5 a 1 mm) y durante tres tiempos de tratamiento (0, 15 y 30 días).

Los mejores resultados se obtuvieron para 15 días de tratamiento con *P. Ostreatus* ya que se obtuvieron mayores niveles de azúcares reductores, actividad enzimática para lacasa (382 U / L), pérdida de lignina y parámetros de Gompertz referidos a la producción de biogás (producción máxima de 302.4 ± 10.7 NmL y una tasa máxima de producción de biogás de 12.2 ± 1.8 d^{-1}), utilizando un tamaño de partícula menor (0.5 a 1 mm).

Estos resultados mostraron que es posible obtener enzimas de alto valor como la lacasa y mejorar la producción de biogás dentro del mismo proceso. Las futuras investigaciones deben centrarse en la evaluación de otras variables tales como la adición de inductores o incluso tiempos de pretratamiento más cortos.

ABSTRACT

This research proposes a new biorefinery concept in which lignocellulosic waste is valorized through a sequential process of fungal treatment and an anaerobic digestion process.

In the fungal treatment stage, the production of high-value enzymes, such as laccase, was studied; in the anaerobic digestion stage, biogas was produced as a renewable energy source.

Wheat straw in two different size ranges (0.5 to 1 mm and 1.4 to 2.36 mm) was subjected to fungal treatment during 30 d to quantify the enzyme production. The degree of enzyme production of three white rot fungi was compared: *Trametes versicolor*, *Pleurotus eryngii* and *Pleurotus ostreatus*. Then, the residual gas production was quantified after 0, 15 and 30 d of fungal treatment.

The best process combination was obtained after 15 d of treatment with *P. ostreatus*, in which higher reducing sugar release, laccase activity (382 U/L), lignin loss and modified Gompertz parameters (a maximum production of 302.4 $\pm$10.7 NmL/g_{VS} and a maximum biogas production rate of 12.2 $\pm$ 1.8 d^{-1}) were obtained for this treatment with smaller particle sizes.

These results showed that it is possible to obtain high-value enzymes, such as laccase, and enhanced biogas production within the same process by approximately 30 %. Further investigations should focus on evaluating other operational variables, such as the addition of inducers and reduced fungal treatment times.

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1. INTRODUCTION

Forest and agricultural industries produce large amounts of residues due to their development. The accumulation of this type of waste, known as lignocellulosic waste (LCW), can lead to different environmental concerns, such as the contamination of large volumes of water and odor and greenhouse-gas emissions (Momoh et al. 2009; Speece, 1996). Hence, it is required to find new strategies to manage these wastes. In addition, the growing energy demand has also imposed the need for developing sustainable energy production processes. Therefore, the application of anaerobic digestion (AD) processes appears to be a viable alternative for using LCWs to produce biogas. AD treatment on LCW is an environmental friendly process with low energy consumption, chemical use and waste production (Bahmani et al. 2016). However, the AD process has different limitations, and therefore, maximizing the economic value of LCWs by producing several valuable products in a biorefinery is crucial (Thomsen, 2005).

In first place, the AD process is complex because several microorganisms participate synergistically through four main biological stages: ***hydrolysis***, in which polymers are degraded to monomers, usually corresponds to the controlling stage for solid substrates, such as LCW; ***acidogenesis and acetogenesis,*** in which volatile fatty acids (VFAs) and acetate are produced; and ***methanogenesis***, in which a biogas mixture is produced (Li et al. 2011). However, the degradation of LCW is a barrier that needs to be overcome in order to make the whole process efficient (Cirne et al. 2007). LCWs are extremely resistant to hydrolysis by AD microorganisms due to their fiber content, which is mainly composed of cellulose, hemicellulose and lignin. Cellulose is characterized as having high strength and flexibility and contains numerous hydroxyl groups that

lead to the formation of a dense and relatively stable polymer (Karaki et al. 2016), while lignin plays the role of cement for the crosslinking between cellulose and hemicellulose, forming the rigid, three-dimensional structure of the cell wall (Palmqvist et al. 2000). Therefore, finding a treatment that could overcome such barrier is crucial.

On the other hand, the initial costs of a biogas plant are rather high. According to reference (Sgroi et al. 2015; Riva el al. 2014) an initial investment of an average of 4 millon euros for a plant with a 999 kW capacity is required. While the production cost are considered low (Riva el al. 2014), this intensive initial investment is a major drawback., thus finding additional valuable products to maximize the value of the residue in order to aid recovering the installation plant costs faster is important.

Since LCWs are composed of carbohydrates making them a suitable feedstock for producing biogas, but represent a barrier to an efficient AD process due to their structure, a treatment prior this processs is usually needed (Mosier et al. 2005). The treatment removes the physical and chemical barriers that make LCW recalcitrant (Jönsson & Martín 2015). Since the accessibility of the polysaccharides greatly improves after pretreating LCW, cost reduction due to enhanced process efficiency or reduced energy requirements is desired (Singh & Singh 2014). There are different types of treatments (physical, chemical and biological) that show diverse results depending on the conditions and substrates used. For example, comminution reduces the particle size and alters the structure of LCW, increasing the accessible surface area (Kratky & Jirout 2011). However, conflicting results have been reported. Applying comminution to oats to decrease the particle size from 2 to 0.5 cm caused no significant differences in the biogas production (Kaparaju

et al. 2002), but decreasing the particle sizes of bagasse and coconut fibers from 5 mm to 0.85 mm led to an increase in the methane yield by 30 % (Kivaisi & Eliapenda 1994). In addition, some research has shown that excessive reduction in the particle size can result in the overproduction of VFAs during AD, inhibiting methane production (Izumi et al. 2010). In contrast, an alkaline pretreatment used to degrade lignin, hemicellulose and cellulose on wheat straw has been shown to increase the methane yield compared to an untreated substrate (Chandra et al. 2012). However, chemical pretreatment can inhibit AD processes, and effluents from the process can lead to environmental issues (Chen 2012). Furthermore, chemical and thermal pretreatments usually require expensive technologies and materials, generating large volumes of waste streams and requiring decontamination of the inhibiting compounds prior to AD (Mosier et al. 2005), which in addition to the high initial cost of a biogas plant make the project unattractive without economic incentives

In contrast, biological treatments, including the use of fungi, microbial consortium and enzymes, to degrade lignin, cellulose and hemicellulose have gained renewed interest (Zheng et al. 2014). While the advantages of biological treatments consist of low energy requirements, reduced waste streams, low downstream costs and reduced inhibitor production for subsequent AD processes (Keller at al. 2003; Nigam & Panley 2009), long retention times and decontamination of the substrate represent major drawbacks for this technology (Taherzadeh & Karimi 2008). Among the three types of biological treatment mentioned, fungal treatment has the advantage of not only degrading the recalcitrant compounds of LCWs, but it also has the capacity of producing an additional high value product such as enzymes.

Fungal treatment involves white, brown and soft rot fungi. While brown fungi mainly degrade cellulose, white and soft rot fungi can degrade cellulose and lignin at the same time. White rot fungi has a high selectivity for lignin and is the most successful (Eriksoon et al. 1990). From the AD process point of view, fungal pretreatments have shown some promising results. For example, it is possible to achieve a release of 22 % of the total sugar from rice straw as monosaccharides (Anasontzis et al. 2017). New research has been developing other uses for fungal pretreatment such as pellet formation improvement (Zhao, 2017).

One example of a white rot fungi that has shown good results as a treatment to degrade the recalcitrant LCWs compounds, corresponds to *Trametes versicolor*, which is a nonselective fungus, meaning it degrades approximately equal amounts of hemicellulose, cellulose and lignin (Hatakka, 2001). Studies have shown lignin loss from corn straw up to 54.6 % after 30 d of pretreatment with *T. versicolor* (Yu et al. 2010b). Another examples correspond to *Pleurotus ostreatus* and *Pleurotus eryngii*. The results of this study indicated lignin loss of a 39.7 % from wheat straw (Zadrazil et al. 1994) and a 33 % increased glucose yield from rice straw while using *P. ostreatus*. Other results have shown a maximum methane yield of 258 L/kg_{VS} after 30 d of fungal pretreatment combined with posterior milling, leading to a 165 % increase in the methane yield compared to untreated rice straw (Mustafa et al. 2016).It has been reported that *P. ostreatus* is more effective on straw materials than other fungi (Taniguchi et al. 2005). However, this fungus is considered to be a moderately selective lignin degrader due to its carbohydrate consumption with long retention times.

The principal mechanism for lignin degradation using white rot fungi mainly involves three enzymes: laccase (Lac), manganese peroxidase (MnP) and lignin peroxidase (LiP) (Ferhan et al. 2013), it is possible to extract these enzymes in order to obtain an additional commercial product. However, not all of these enzymes are found in every fungal culture. Genes encoding lignolytic enzymes from white rot fungi are differentially regulated by several environmental signals (Janusz at el. 2013). For example, LiP gene expression is dramatically regulated by environmental conditions, such as nitrogen-deficiency (Stewart et al. 1992).

Although, the three fungi mentioned (*T. versicolor*, *P. eryngii*, and *P. ostreatus*) have shown high capacity to treat LCW prior AD, the enzyme production of these fungi is different. While *P. eryngii*, as well as *P. ostreatus*, is a representative specie from the Lac-MnP enzyme production group, *T. versicolor* has been reported to produce the three enzymes: Lac, MnP and LiP (Hatakka, 2001).However, since decontamination is one of the major costs for fungal pretreatment in scaled-up applications (Forough et al. 2013), its effect on the overall process of applying a fungal treatment followed by the AD should be evaluated. Akhtar et al. (1998), reported that complete decontamination was not always necessary for fungal pretreatment with white rot fungi. Previous results have shown that white rot basidiomycetes, such as *Phanerochaete chrysosporium*, are not highly affected by other fungi or bacterial infections, while *Ceriporiopsis subvermispora* has been reported to be more vulnerable (Srebotnik et al. 1994).

As mentioned before, white rot fungi have the ability to produce Lac and MnP enzymes from LCWs such as wheat straw. During the process to obtain biogas, applying AD on LCWs may benefit from these enzymes, and there is also a commercial use for both of them; thus, recovering

these enzymes may add value to the process. Laccases have the ability to oxidize phenolic and nonphenolic lignin related compounds as well as highly recalcitrant pollutants (Couto & Herrera 2006). The application of these enzymes varies from the food industry to the pulp and paper industry, yet the oldest and largest industrial application corresponds to dye bleaching in the textile industry (Riva 2006). In contrast, MnP is the key enzyme under current investigation for pulp biobleaching (Isroi et al. 2011). Despite the promising industrial applications of these enzymes, certain obstacles, such as the lack of commercial stock and the high cost of redox mediators, need to be overcome. For these reasons, the proposal of a new strategy to maximize the value of residues by producing biogas and other by-products, such as enzymes, may be promising.

2. OBJECTIVES

The objective of this work is to propose a biorefinery strategy that applies a sequential process that includes a physical/fungal treatment followed by anaerobic digestion. The main goal of the process is to maximize the value of industrial residues by producing enzymes, such as laccase and manganese peroxidase, and biogas, as a renewable-energy source.

The study evaluates the effects of three different white rot fungi, *Trametes versicolor*, *Pleurotus ostreatus and Pleurotus eryngii*, on an abundant LCW, such as wheat straw.

Finally, two operational variables are studied: the particle size of the residue and pretreatment time.

3. MATERIALS AND METHODS

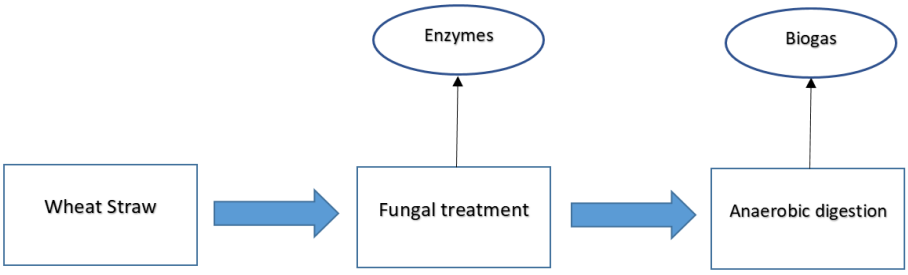

Figure 1. Sequential process applied to wheat straw and its products.

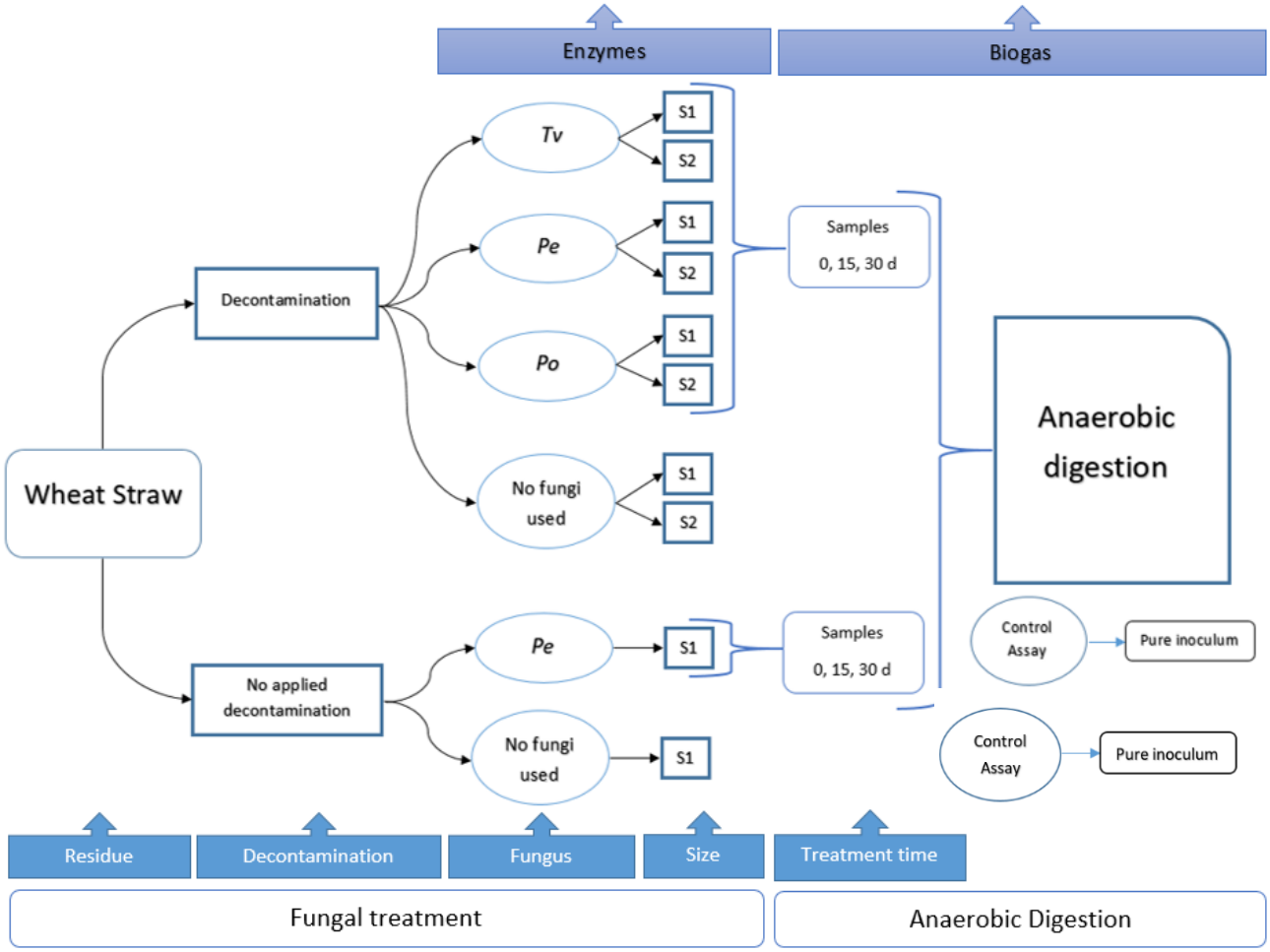

Figure 2. *Experimental design diagram.*

3.1. Materials

Residue

LCW wheat straw was used as the raw material. It was obtained from a farm located in Lampa, Santiago, Chile. It was characterized by total solid (TS), volatile solids (VS) and total lignin (TL) content (table 1). It was chopped into sizes under 5 mm. The particle size was determined by sieving (ASTM E-11) using an SS-15 model sifter (Gilson Company Inc). Two levels of particle sizes were used: **S1** between 1.4 and 2.36 mm and **S2** between 0.5 and 1 mm.

Figure 3. Wheat straw used for the study.

Table 1. Wheat straw characterization.

	TS [%]	[g VS/g TS]	TL [%]
Wheat straw	92.2	0.9	26

Fungi

The three white rot fungi, *Trametes versicolor (Tv), Pleurotus ostreatus (Po) and Pleurotus eryngii (Pe)*, were obtained due to a donation from CIB located in Madrid, Spain. They were incubated in agar petri dishes for blank seven days at 30 °C in a MEA medium. After this process, submerged

cultures were made by adding 100 mL of the liquid medium (Salvachúa et al. 2011) into 250 mL Erlenmeyer flasks. These cultures were maintained at 30 °C and 150 rpm in a shaker for two weeks. Finally, the excessive water was removed, and the fungi pellets (FPs) were stored at 4 °C.

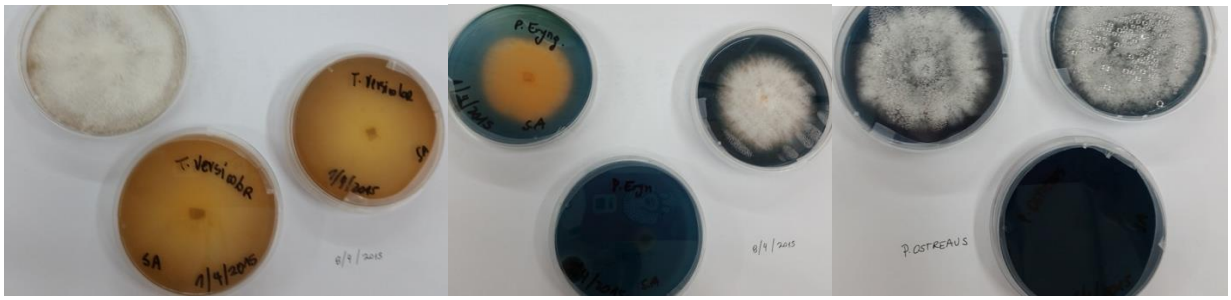

Figure 4. Agar petri dishes for the three different fungi used in the study: *Trametes versicolor, Pleurotus eryngii and Pleurotus ostreatus.*

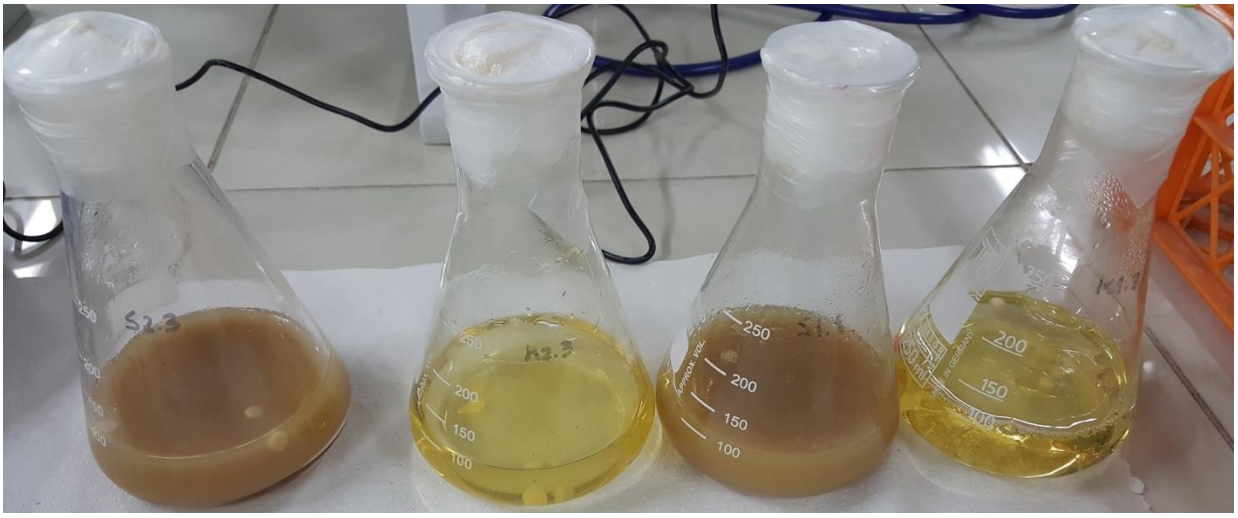

Figure 5. Submerged cultures growth in liquid medium.

Inoculum

The inoculum was obtained from the company Ecoriles S.A. located in Santiago, Chile. It was concentrated by removing excess water, then it was characterized by chemical oxygen demand (COD), total and volatile solid content.

Table 2. Inoculum characterization.

	[g VS/g]	[g TS /g]	[g COD/g]
Inoculum	0.042	0.052	0.055

3.2. Experimental methods

Decontamination

Decontamination was applied to the raw material prior to fungal treatment in order to isolate the fungus effect. This treatment was conducted using an autoclave with a cycle of 30 minutes at 121 °C for all samples used in the study. However, to elucidate the effect of decontamination on the AD, a single study using size S1 and *Pe* fungus was not subjected to decontamination (figure 2).

Fungal treatment

For the study, every assay contained FPs and wheat straw in a ratio of 2 mL/g. In addition, water was added to maintain the moisture above 60 %, but free water was not present in the flasks in order to conduct solid substrate fermentation. The 250 mL flasks were kept in a static incubator at 30 °C. Every 4 days, three flasks were removed to measure their enzymatic activity and reducing sugar content. In parallel, samples of 0, 15 and 30 d (figure 2) of pretreatment were obtained to apply AD process on them On the other hand, control assays were performed to confirm that substrate did not show any enzymatic activity before and after the decontamination process. In addition, the total solids and lignin content was quantified in the samples after 0, 15 and 30 d of treatment. The results are reported as total solids loss (TS_{loss}) and total lignin loss (TL_{loss}) according to:

$$TS_{loss} = \frac{TS_0 - TS_x}{TS_0} * 100\ [\%] \quad (1)$$

$$TL_{loss} = \frac{TL_0 - TL_x}{TL_0} * 100\ [\%] \quad (2)$$

Where TS_0 correspond to initial total solid content, TL_0 correspond to initial total lignin content and TS_x/ TL_x correspond to the total solid and lignin content for x equal to 15 and 30 d of treatment.

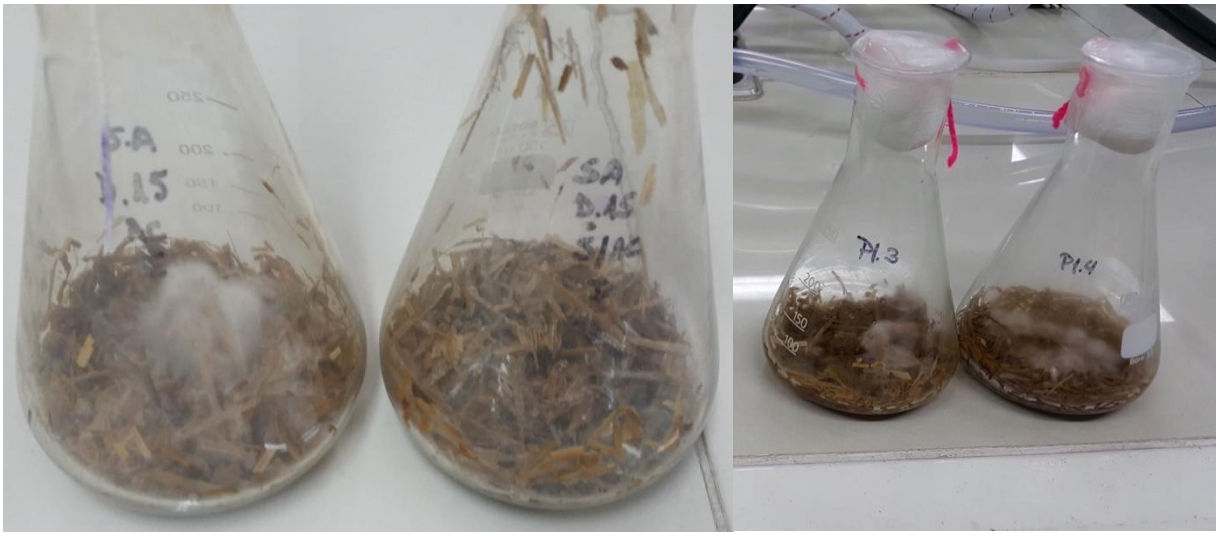

Figure 6. Fungal growth on wheat straw in a solid-state culture.

Anaerobic digestion

The biogas potential batch tests were performed using samples taken from the fungal treatment according to Holliger et al. (2016). These samples contained substrate and fungus, which was allowed to grow for 0, 15 and 30 d. These assays were carried out using 250 mL Pyrex bottles with a total solids content of 18 %, pH between 7.0 and 7.2, and a substrate/inoculum ratio of 1 g_{VS}/g_{VS}. In addition, macronutrients, micronutrients and buffer solutions were added according to

Angelidaki et al. (2009). Every bottle was flushed using N_2 to remove the air from the headspace and sealed with rubber stoppers. Finally, the assays were kept at 30 °C in a hot chamber with continuous agitation at 160 rpm in a roller bottle apparatus (Wheaton). The temperature chosen was according to the optimal growth temperature for the fungal treatment considering that the sequential process was going to be applied within the same structure.

The biogas production was measured using a pressure transducer (IFM), with a range between 10-1000 mbar. The results are presented as biogas production per g of VS under normal conditions of pressure and temperature (273 K and 1 bar) after substracting the control (figure 2).

Figure 7. Pressure transducer used (left) and anaerobic digestion assay (right).

Chemical Analysis

For fungal treatment characterization, the enzymatic activities were quantified from extracts of the fermented substrate. For extracellular compound extraction, 50 mL of water was added to every sampled flask. This product was manually homogenized; and then it was mixed for 2 hours at 200 rpm in a thermostatic shaker (JS Research Inc.) at 20 °C. The flask content was centrifuged for 5

minutes at 5000 rpm, and supernatant samples were stored for characterization. The laccase (Lac) activity was measured using the ABTS method at a wavelength of 436 nm with a molar extinction coefficient of 29.300 $M^{-1}cm^{-1}$ (Niku-Paavola et al. 1990). The manganese peroxidase (MnP) activity was measured using the DMP oxidation method at a wavelength of 469 nm with a molar extinction coefficient of 27.500 $M^{-1}cm^{-1}$ (Martínez et al. 1996). In addition, the reducing sugar was measured using the DNS method (Miller 1959). The soluble and insoluble lignin content was quantified according to the NREL (Sluiter et al. 2012). The total solids (TS) and volatile solids (VS) contents were measured according to the standard methods (APHA, AWWA, WPCF, 2005).

Statistical Analysis

To evaluate the biogas production, the modified Gompertz equation was used to model the accumulated biogas production ($B(t)$) with time (t) (Altas 2009; Lo et al. 2010).

$$B(t) = A \cdot exp\left(-\exp\left(\frac{\mu \cdot e^1}{A} \cdot (\lambda - t) + 1\right)\right) \qquad (3)$$

The kinetic constants of the equation are as follows: A (biogas production potential[NmL/g_{VS}]), μ (maximum biogas production rate [$NmL/g_{VS}d$]) and λ (lag phase period [d]). They were estimated using a nonlinear regression approach using the solver command in Microsoft Excel®. All assays were conducted in triplicate, from which the average biogas production ($\overline{y}$) and standard deviation (s) were calculated. In addition, the confidence interval was calculated within 95 % certainty using a t-test $\left(t_{\alpha/2,v}\right)$.

$$y_{sup/inf} = \overline{y} \pm t_{\frac{\alpha}{2},v} s \sqrt{\frac{1}{s}} \qquad (4)$$

Biogas potencial

The theoretical biogas potential was estimated using equation (5), (Kaparaju et al. 2010; Kaparaju et al. 2009). The values of carbohydrates, protein and lipids for raw wheat straw according to Kaparaju et al. (2009), were used for the estimation, considering reference values with a similar total solid and lignin content, while the values of acetate and propionate were not considered for the estimation.

$$B_t = \frac{(0.415\ Carbohydrates + 0.496\ Proteins + 1.014\ Lipids + 0.373\ Acetate + 0.530\ Propionate)}{(\ Carbohydrates + \ Proteins + \ Lipids + Acetate + \ Propionate)} \quad (5)$$

Where B_t corresponds to the theoretical biogas production $[m^3/g\ VS]$.

4. RESULTS AND DISCUSSION

4.1. Fungal Treatment

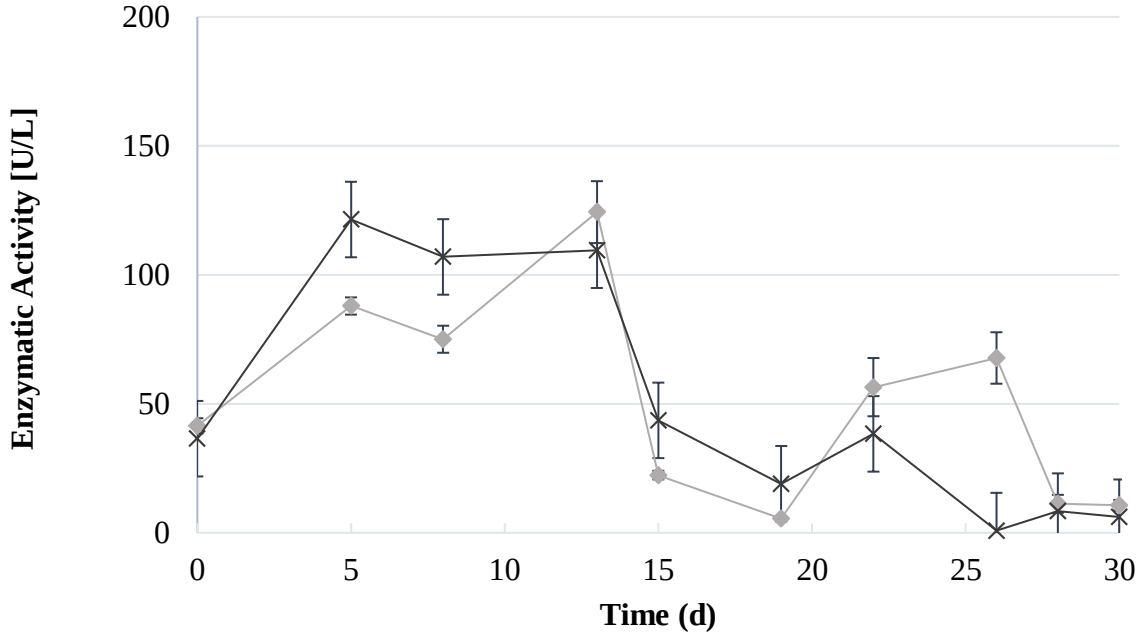

Figure 8. Laccase activity for the *Tv* treatment on wheat straw for sizes S1 (◆) and S2 (✕).

The goal of the fungal treatment studied in this work was to produce enzymes, combined with the subsequent effects that occur during the AD process. Then, the enzyme production of Lac and MnP using the three different fungi with wheat straw as a nutrient was evaluated. The laccase activity (figure 7) for the *T. versicolor* treatment had a peak of enzymatic activity for both sizes S1 and S2 between days 0 and 15. These results are consistent with other studies in the literature, which also showed maximum laccase activity between 0 to 15 d for fungus growth in a synthetic medium (Ryan et al. 2006). After day 15, the laccase activity declined. Furthermore, a significant difference, up to 40 %, between the results for S2 and S1 was measured after the 10th day,

indicating that S2 obtained higher laccase activities. In contrast, after 15 d of treatment, the differences obtained for both size were negligible.

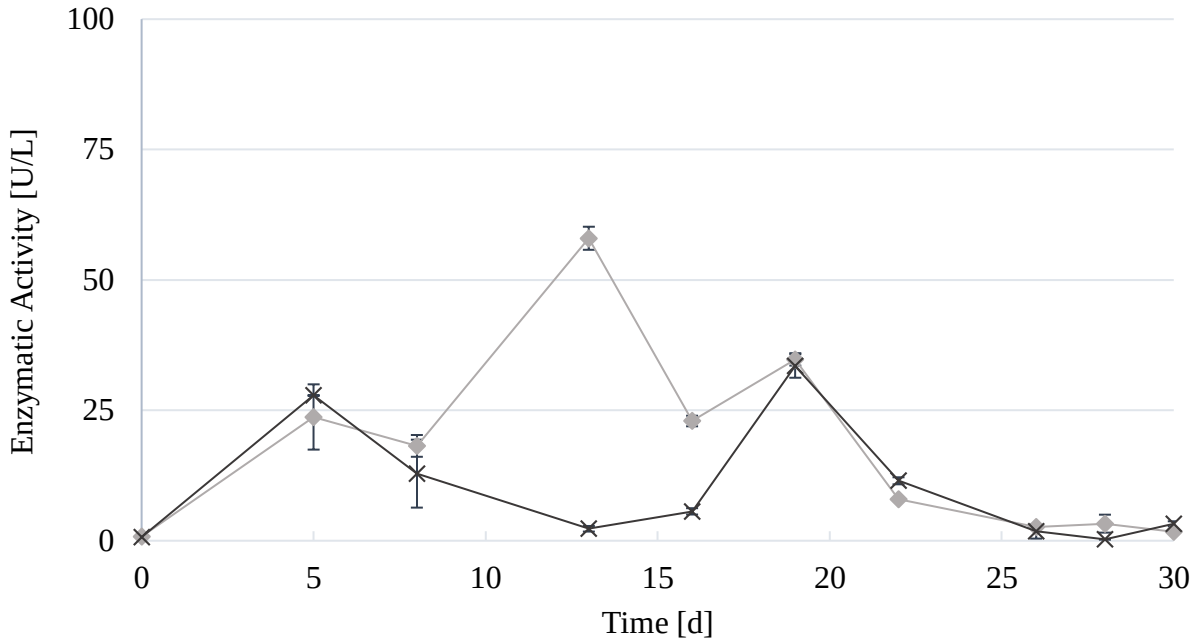

Figure 9. Laccase activity for the *Pe* treatment on wheat straw for sizes S1 (◆) and S2 (✕).

The treatment carried out with *P. eryngii* (figure 8) produced extremely low laccase activity during the whole treatment time, implying that the experimental conditions used, i.e., the substrate-fungus combination, were not favorable for this enzyme production.

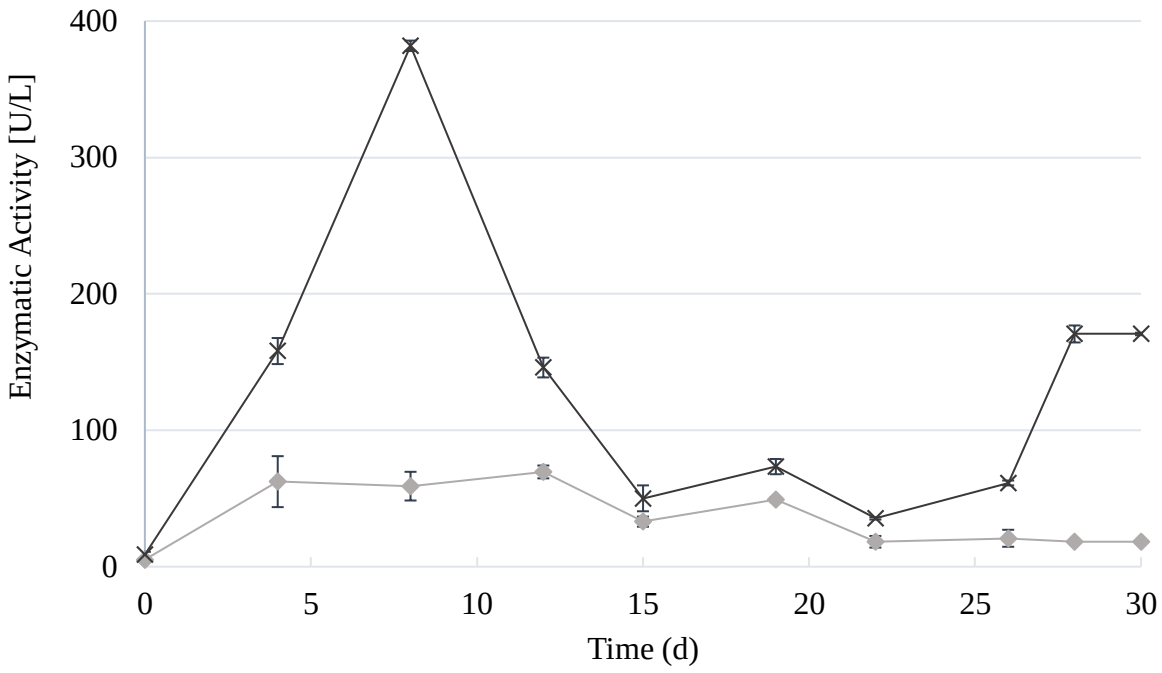

Figure 10. Laccase activity for the *Po* treatment on wheat straw for sizes S1 (—◆—) and S2 (—×—).

A completely different result was obtained during the *P. ostreatus* treatment (figure 9). This fungus reached a maximum laccase activity between 0 to 8 d, with significant differences between the S1 and S2 behavior. For the bigger particle size (S1), the laccase activity was rather low. For the smaller particle size (S2), there was a peak at approximately 382 U/L in the laccase activity at 8 d of treatment, after which time the laccase activity decayed quickly. The behavior observed could be explained by the smaller particles sizes having an increased superficial area, while the larger particle sizes hampered the penetration of the fungus and AD microorganisms (Reid, 1989).

Since the laccase enzyme is able to oxidize lignin, it plays a significant role during the whole lignin degradation process (Thurston, 1994). For that reason, a high laccase activity is indicative of a highly active degradation process of the substrate, in this case the wheat straw. According to the literature, it is also possible to induce laccase production by the addition of Cu^{+2} (Palmieri et al.

2000). An addition of 25.0 mM of Cu^{+2} has been reported to enhance laccase activity from 270 to 1420 U/L when using *Pleurotus pulmonarius* (Tychanowicz et al. 2006). However, conflicting results have been reported. According to Stajic et al. (2006), an addition of 1 mM is the optimal dose for enhancing laccase production with *P. ostreatus.* In contrast, Patel et al. (2009) reported that an addition of over 0.3 mM of Cu^{+2} could inhibit fungal growth and therefore decrease the laccase activity.

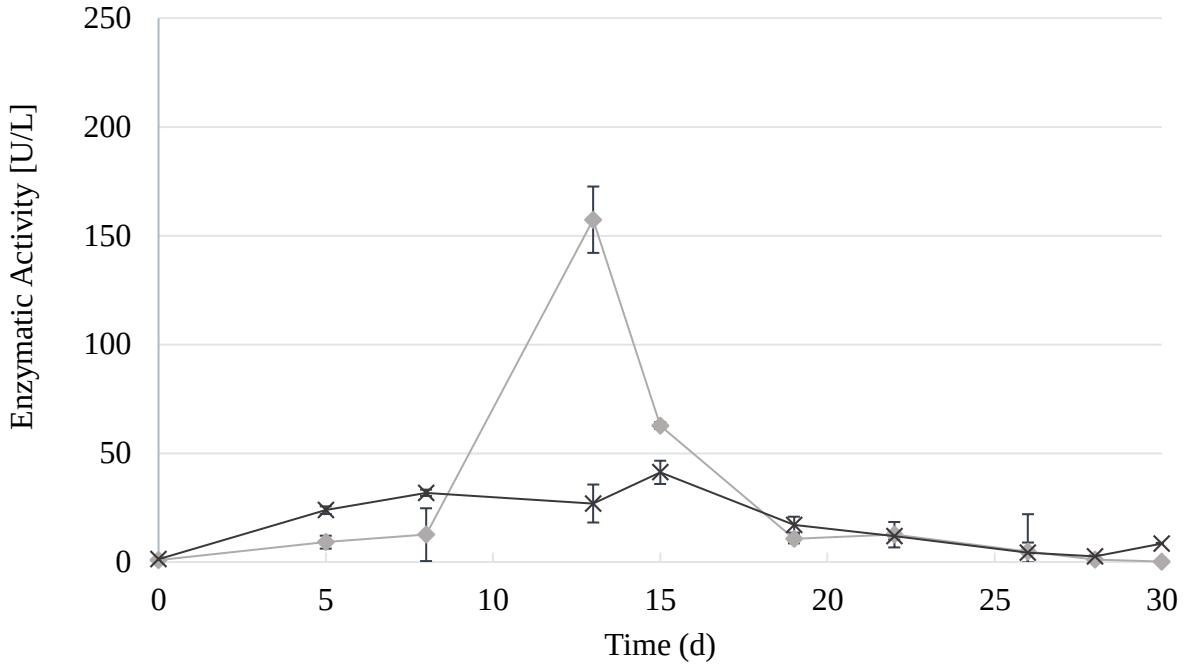

Figure 11. Manganese peroxidase activity for the *Tv* pretreatment on wheat straw for sizes S1 (◆) and S2 (✕).

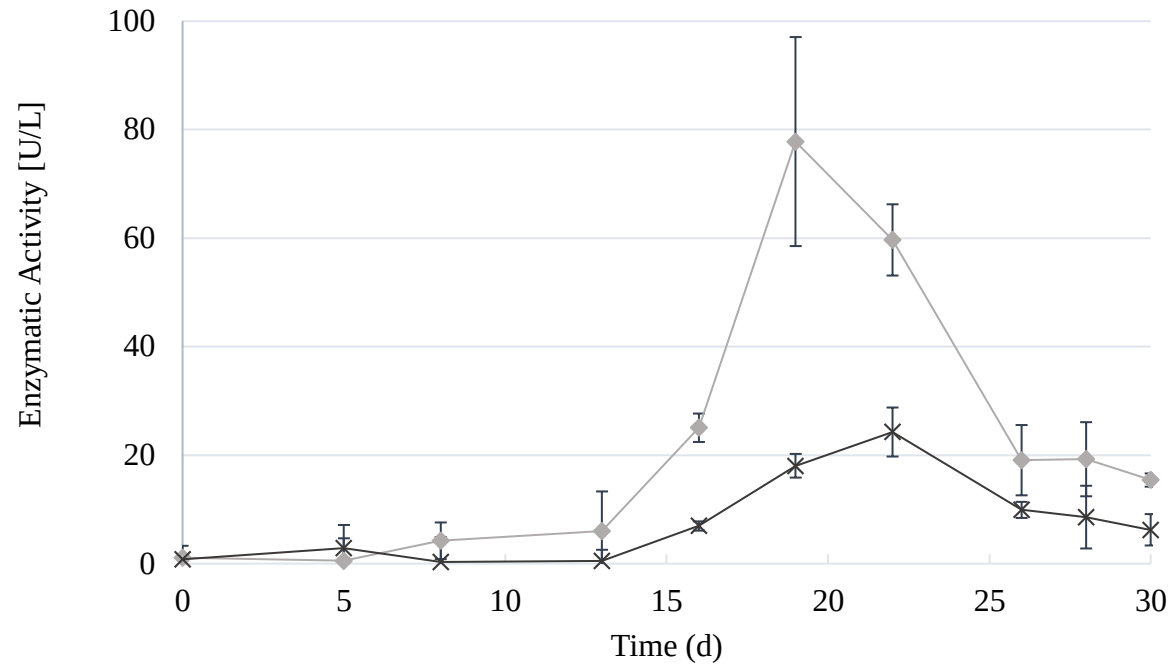

Figure 12. Manganese peroxidase activity for the *Pe* pretreatment on wheat straw for sizes S1 (—◆—) and S2 (—×—).

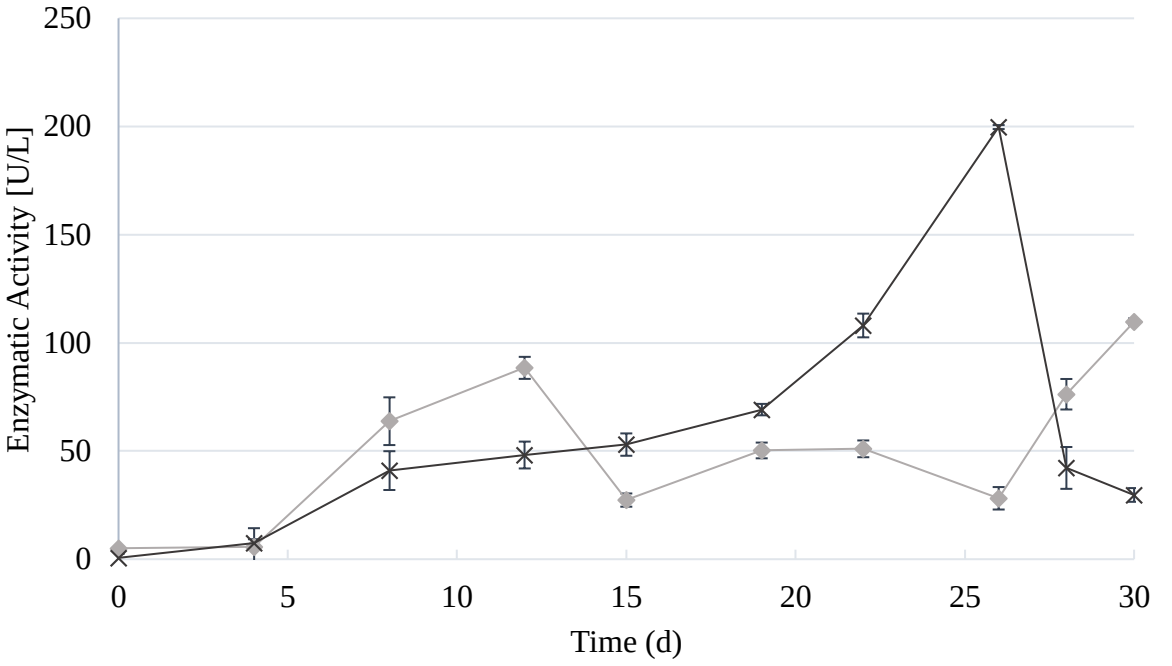

Figure 13. Manganese peroxidase activity for the *Po* pretreatment on wheat straw for sizes S1 (—◆—) and S2 (—×—).

For the MnP enzyme production, the results obtained were different than those obtained for the Lac production. Significantly different behavior responses were obtained for every fungal treatment, while *T. versicolor* (figure 10) reached a maximum MnP activity during the first stage of the pretreatment (0 to 15 d), the maximum peaks for *P. eryngii* (figure 11) and *P. ostreatus* (figure 12) occurred during the last stage (15 to 30 d). Additionally, a comparison of the different sizes showed that for *T. versicolor* and *P. eryngii*, S1 enabled higher MnP production: 157 U/L and 78 U/L, respectively. In contrast, *P. ostreatus* enabled higher production using the smaller particle size (S2) with 200 U/L. Manganese peroxidase is an enzyme that is secreted to aid lignin degradation by catalyzing the oxidation of phenolic compounds that can inhibit AD (Wan et al. 2011). MnP gene expression is generally regulated by complex culture conditions, such as the presence of Mn^{+2} (Janse et al. 1998). However, it has been reported that the presence of Mn^{+2} is not always a defining factor for *P. ostreatus*. A pretreatment of observed dust with the use of this fungus indicated that MnP gene expression was not regulated by the presence of Mn^{+2} (Giradina et al. 2003). This implies that *P. ostreatus*, unlike other fungi, can produce MnP under several conditions.

To summarize the fungal treatments for enzyme production, the fungus *P. ostreatus* was the best enzyme producer for this lignocellulosic waste compared to *P. eryngii* and *T. versicolor*. This fungus showed the ability to secrete high amounts of both enzymes, laccase and manganese peroxidase, during different time points of its growth. The Lac enzyme was produced during the first stage (before 15 d), and the MnP enzyme was produced after 15 d. From this point of view, it would be possible to control the enzyme production by varying the incubation time.

The enzymes secreted during the fungal treatment are responsible for releasing organic matter from the solid substrate to the extracellular media to be used as nutrients for fungal growth. From this point of view, we quantified the amount of reducing sugars available during the fungal treatment in order to predict if they would be available for the anaerobic digestion step.

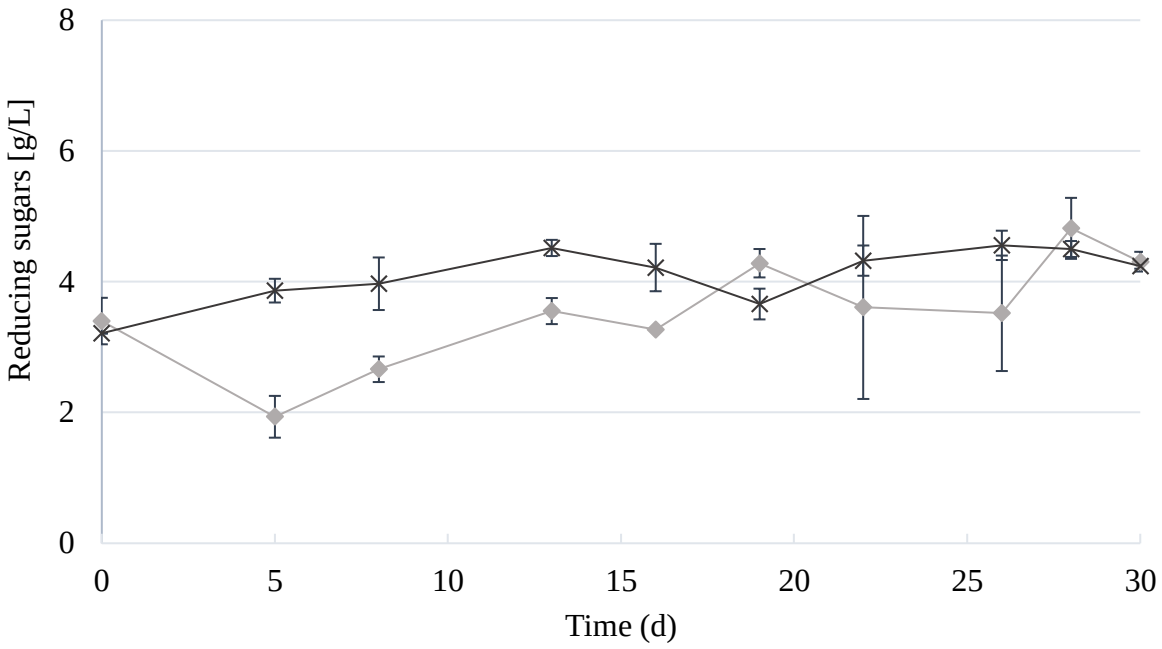

Figure 14. Reducing sugars for the *Tv* treatment on wheat straw for sizes S1 (—◆—) and S2 (—×—).

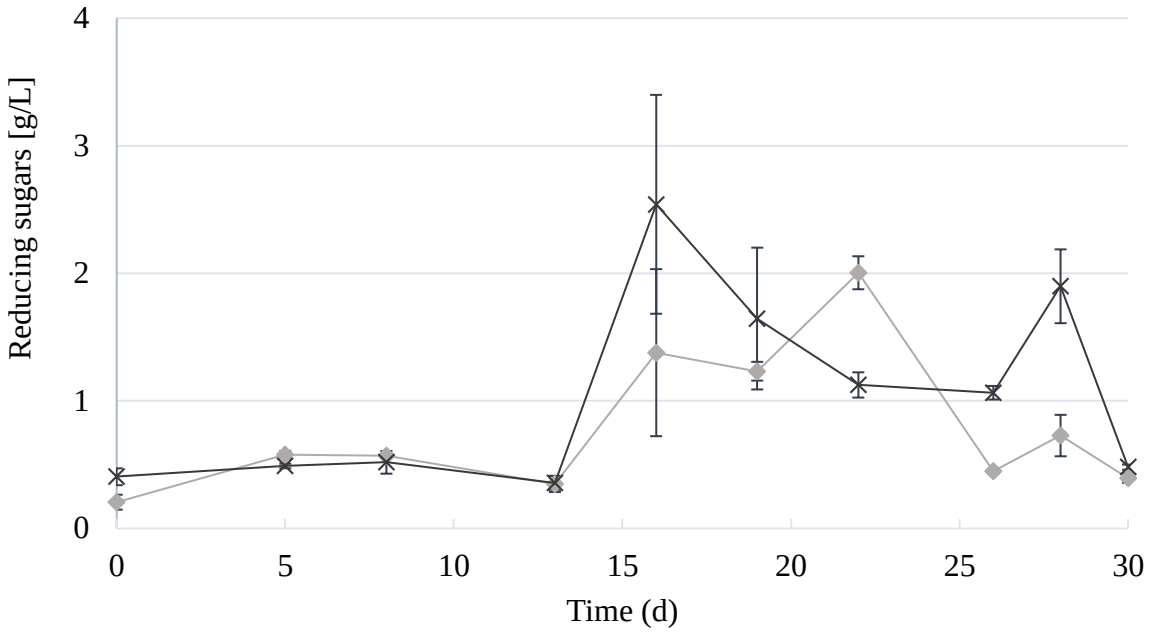

Figure 15. Reducing sugars for the *Pe* treatment on wheat straw for sizes S1 (◆) and S2 (×).

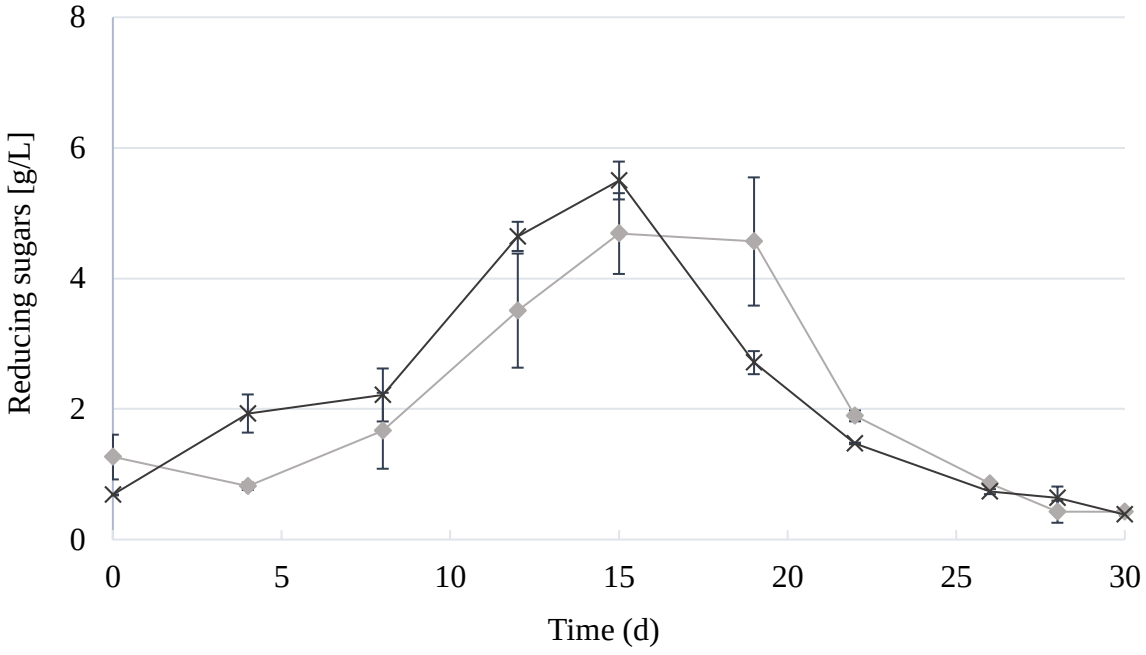

Figure 16. Reducing sugars for the Po treatment on wheat straw for sizes S1 (◆) and S2 (×).

The comparison of the reducing sugar content in the extracts of the fungal-treated wheat straw using different fungi indicated completely different behaviors. In the particular case of *T. versicolor* (according to figure 13), the reducing sugar behaved in a different way for both sizes of waste studied (S1 and S2). While the maximum concentration peak detected for S2 occurred between day 0 and 15, the maximum peak for S1 occurred between day 15 and 30, meaning that the particle size between 0.5 and 1 mm increased the release of reducing sugars by approximately 29 % during the first 13 d. Furthermore, these results indicated that after the maximum peaks we achieved, the quantity of reducing sugars released to the external medium was maintained at the same or lower value, probably due to the fungus growth. *T. versicolor* is considered to be a nonselective fungus, which means that it can degrade cellulose and lignin at the same time. This also means that *T. versicolor* usually consumes carbohydrates faster than other more specific fungi. These results are consistent with the results obtained for the fungal pretreatment of rubberwood with *T. versicolor,* which showed only a 6 % increase in the reducing sugar yield during 30 d of pretreatment (Forough et al. 2013). According to the literature, higher selectivity is associated with better results for delignification, and lower selectivity indicates high cellulose loss during fungal treatment (Camarero et al. 1994).

A different behavior for the extractable reducing sugars was obtained using *P. eryngii* (figure 14), in which there was an almost negligible release during the first days of pretreatment, and after thirteen days, the concentration increased slightly. As mentioned in the introduction section, in this proposed sequential process, it is desirable to use a fungus that requires a low consumption of sugars for its growth. While *P. eryngii* has been reported to be a selective lignin degrader (Valmaseda et al. 1990; Valmaseda et al. 1991), it has also been reported to be a lower reducing

sugar releaser (Isroi et al. 2011). Although lignin degradation might be essential, it is not always positively correlated to higher cellulose and hemicellulose digestibility (Capelari & Tomás-Pejo 1997, Yu et al. 2010a). The differences between the sizes were not significant during the early stage of pretreatment (0 to 15 d). Later on, the release of the reducing sugars varied. The release was higher for S1 at first but became higher for S2 closer to 30 d of treatment.

In the case of *P. ostreatus* treatment, Figure 15 revealed that it was capable of releasing more reducing sugars than both of the other fungi studied before 15 d of pretreatment. Consequently, after this time limit, there was a strong decay in the reducing sugar concentration of the extract, implying its consumption by the fungus. The behavior observed means that the consumption of carbohydrates from *P. ostreatus*, a semiselective fungus, increased with longer treatment times, in agreement with the reported literature (Wan et al. 2012). Considering that the success of the subsequent AD process depended on the ability to release the compounds trapped in the extracellular matrix, thereby increasing the concentration of the reducing sugars, the treatment studied should last 15 d at most using *P. ostreatus*. Similar behavior was observed previously for laccase production (Galhaup & Haltrich. 2001).

Another expected effect due to the enzyme action was the variation in the total lignin and total solids content (Table 1). The quantification of these variables showed that the three fungi reduced the lignin content of the wheat straw for the smaller particle size (S2). According to previous results regarding reducing sugars and enzymatic activity, a difference was also a observed based on the treatment time. Higher amounts of reducing sugars were released and enhanced Lac activity was obtained between 0 to 15 d of treatment rather than the 15 to 30 d period, which only exhibited

higher MnP activity. This behavior was also seen for the lignin loss. Lignin losses of approximately 13.1 and 16.7 % were obtained during treatment with *P. ostreatus* after 15 d. However, lignin losses of only approximately 6.4 and 17.3 % were obtained at 30 d for S1 and S2, respectively. Even when higher lignin loss was obtained after 30 d of fungal treatment, in the case of the smaller particle size (S2), the difference between 15 and 30 d was rather low, which in addition to the previous results, indicated that the optimal time is during the early stage of the treatment (0 to 15 d). The results for *P. ostreatus* are similar to those that have been reported previously for ethanol production applications, which showed lignin losses of 2, 18 and 27 % after 7, 14 and 21 d of treatment with *P. ostreatus* after a mild alkali pretreatment on wheat straw (Salvachúa et al. 2011). However, in this study, it was possible to obtain similar delignification results after 15 d of treatment without alkali pretreatment applied. In contrast, the total solids content loss provided an estimate of the substrate degradation due to the fungus during treatment. The highest TS loss was obtained after 30 d of treatment with *T. versicolor*, while the lowest TS loss was obtained after 15 d of treatment with *P. eryngii*, both for smaller particle sizes (S2).

Table 3. Total solids (TS) and total lignin (TL) loss after 15 and 30 d of fungal treatment (±standard deviation).

		TS loss [%]		**TL loss [%]**	
		15 d	**30 d**	**15 d**	**30 d**
T. versicolor	S1	11.7±0.2	30.0±0.1	3.1±0.2	5.5±0.1
	S2	16.9±0.1	33.0±0.0	6.1±0.1	13.7±0.0
P. eryngii	S1	7.0±0.1	7.6±0.1	2.8±0.0	5.3±0.0
	S2	6.0±0.1	7.6±0.1	7.6±0.2	9.5±0.1
P. ostreatus	S1	18.3±0.0	19.2±0.1	13.1±0.1	6.4±0.0
	S2	9.7±0.1	26.4±0.1	16.8±0.1	17.3±0.1

4.2. Anaerobic digestion

Previous results in the literature showed that the application of a fungal treatment prior to different processes, such as anaerobic digestion or fermentation, can augment the availability of organic compounds for subsequent processes (Forough et al. 2013; Yu et al. 2009; Hatakka. 1983; Taniguchi et al. 2005). However, those studies never focused on the availability of the enzymes produced, and also, they were not able to indicate if the increase in the enzyme production could increase or decrease the biogas production.

The results described in figure 16 to 21 indicate a priori that there were not significant differences between most of the fungal treatments studied. However, the theoretical biogas potential estimated according to equation (5), corresponds to 415 NmL/ g VS which is higher in more than a 30 % to any of the maximum biogas potential obtained in any case of the study.

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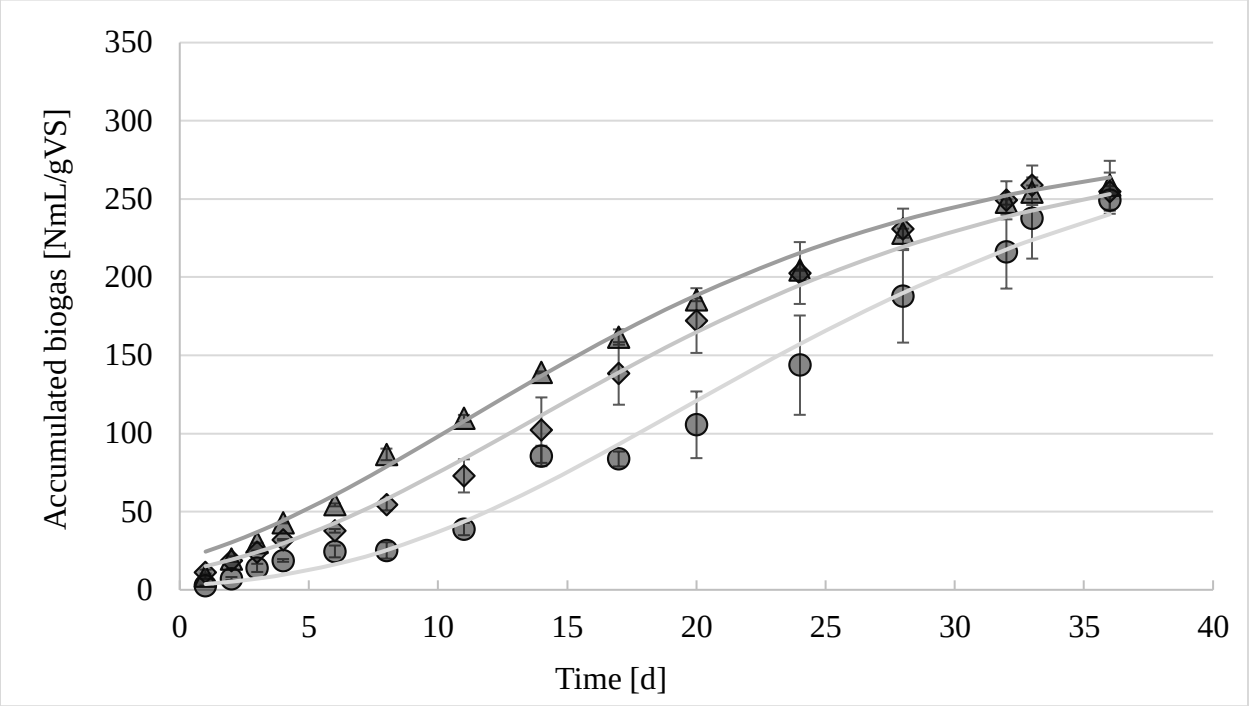

Figure 17. Biogas production per volatile solids content after *Tv* treatment on wheat straw for size S1 after 0 (▲), 15 (◆) and 30 (●) days, where the solid line represents the modified Gompertz model curve.

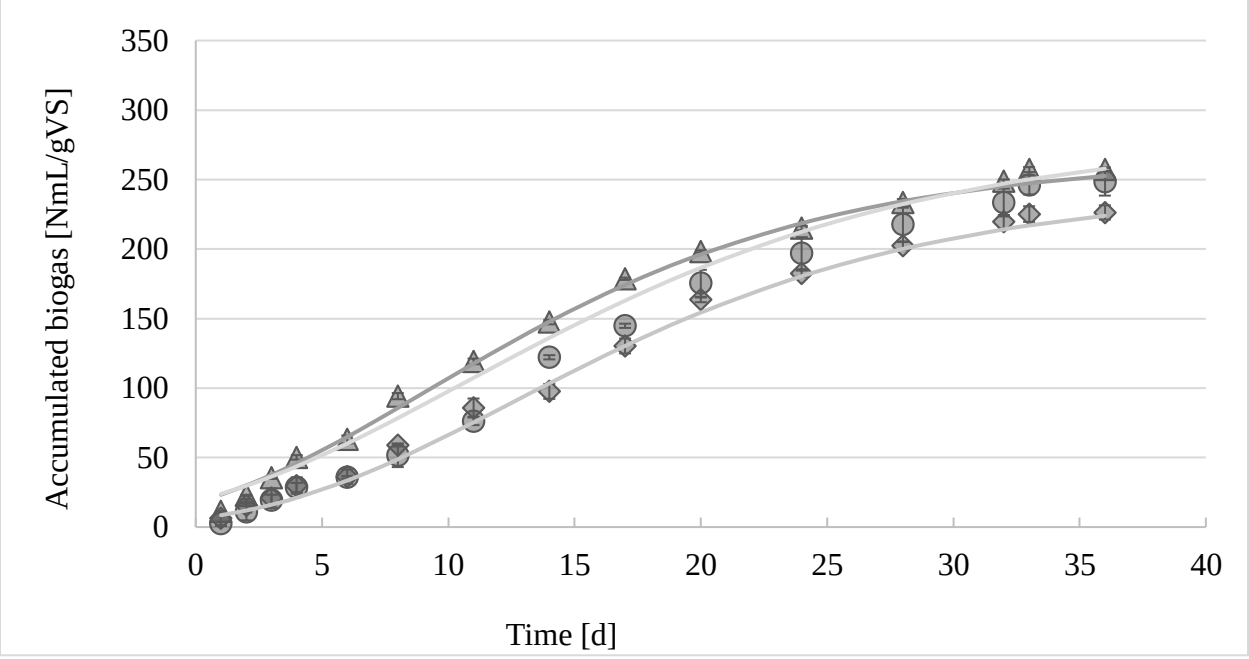

Figure 18. Biogas production per volatile solids content after *Tv* treatment on wheat straw for size S2 after 0 (▲), 15 (◆) and 30 (●) days, where the solid line represents the modified Gompertz model curve.

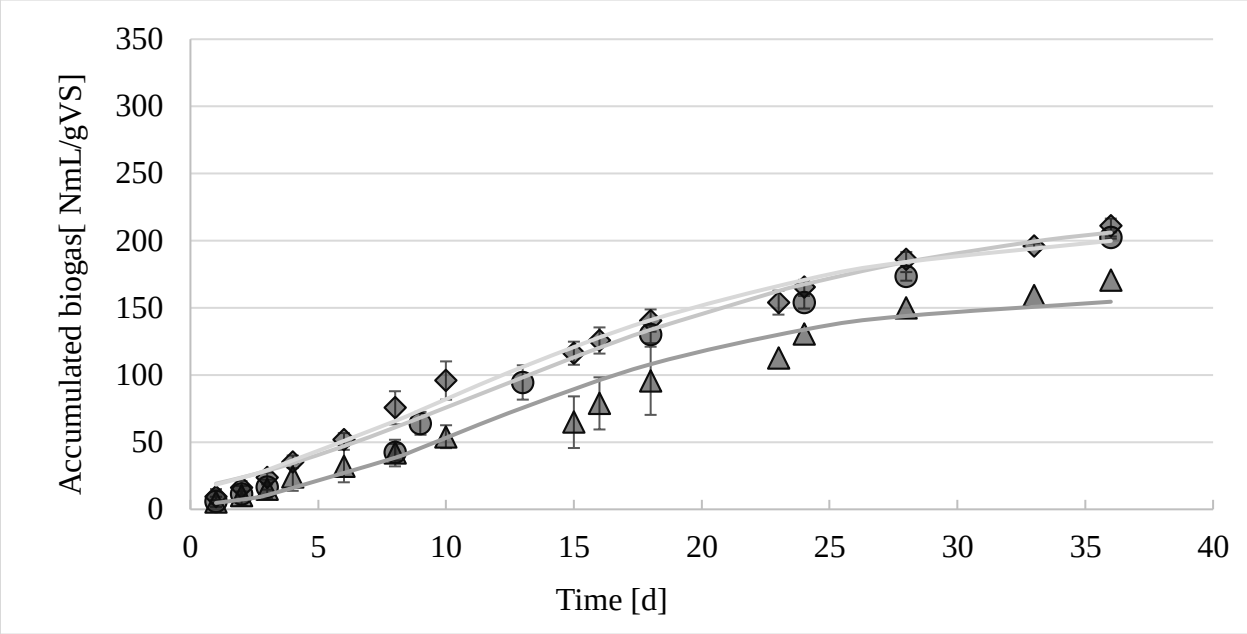

Figure 19. Biogas production per volatile solids content after *Pe* treatment on wheat straw for size S1 after 0 (▲), 15 (◆) and 30 (●) days, where the solid line represents the modified Gompertz model curve.

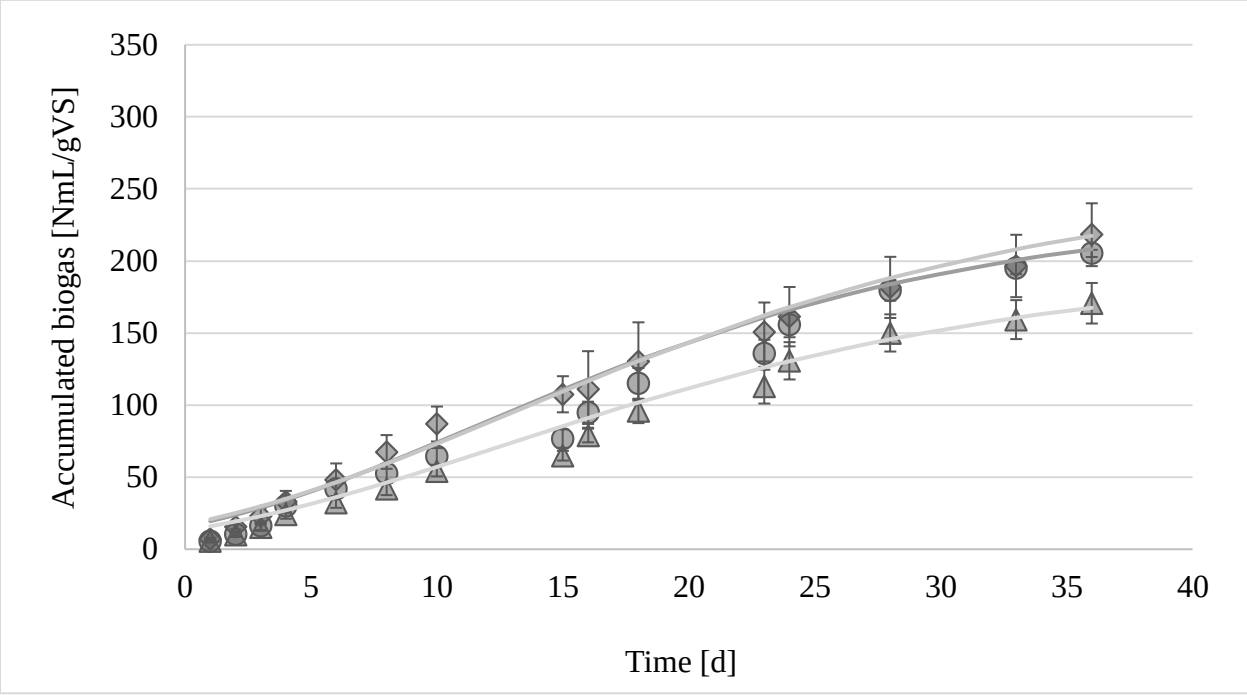

Figure 20. Biogas production per volatile solids content after *Pe* treatment on wheat straw for sizes S2 after 0 (▲), 15 (◆) and 30 (●) days, where the solid line represents the modified Gompertz model curve.

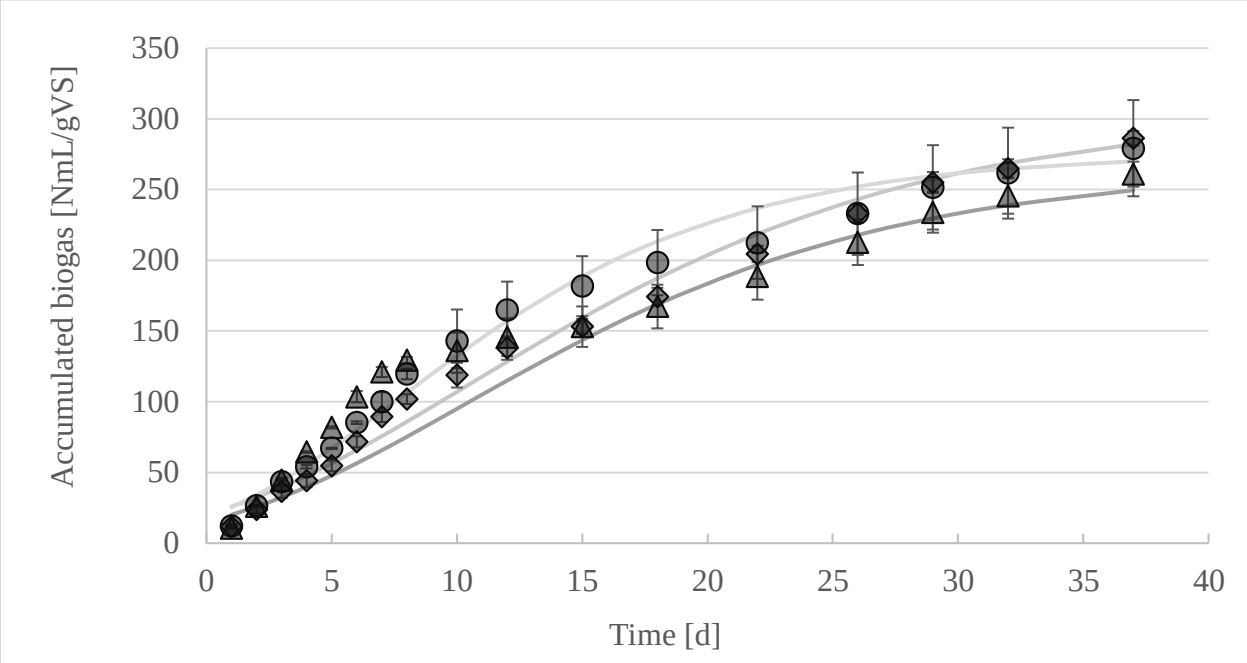

Figure 21. Biogas production per volatile solids content after *Po* treatment on wheat straw for sizes S1 after 0 (▲), 15 (◆) and 30 (●) days, where the solid line represents the modified Gompertz model curve.

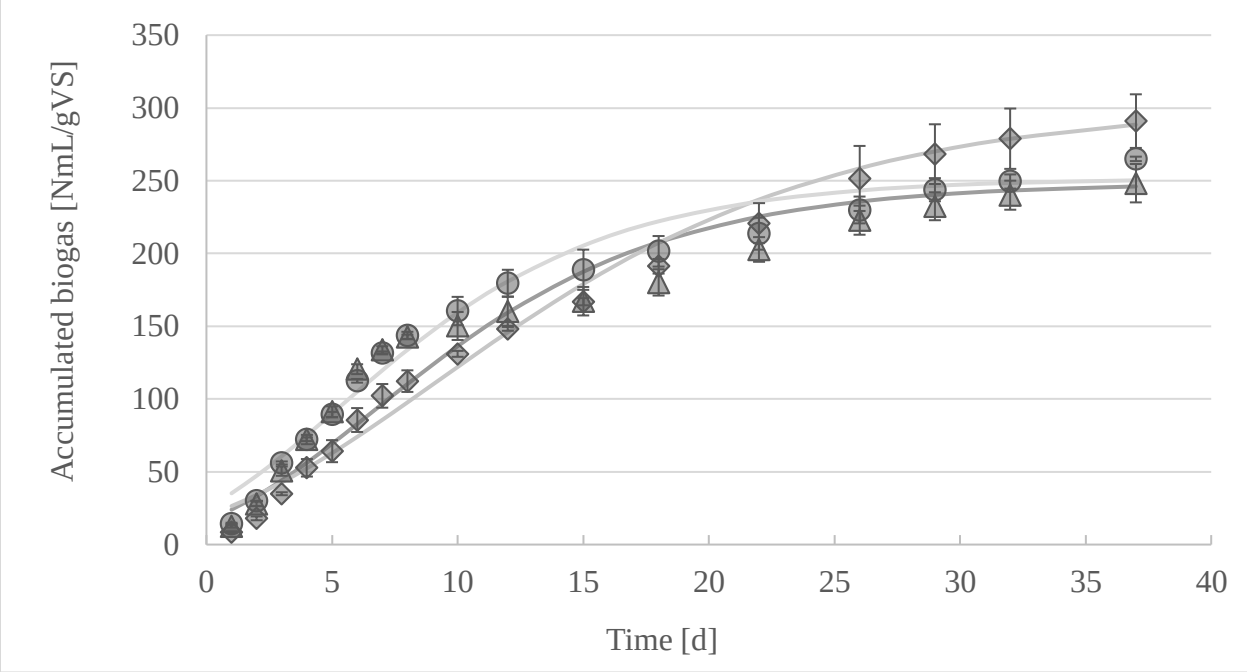

Figure 22. Biogas production per volatile solids content after *Po* treatments on wheat straw for sizes S2 after 0 (▲), 15 (◆) and 30 (●) days, where the solid line represents the modified Gompertz model curve.

For the treatment with *T. versicolor*, the fungal treatment curve after 0 d of treatment is higher than the curves corresponding to 15 and 30 d for both sizes studied (figure 16 and 17), implying that negligible biogas production occurred due to fungal growth. However, this result is in agreement with the nonselective fungus behavior of *T. versicolor* described in the fungal treatment section. The treatment with *P. eryngii* for both sizes (figure 18 and 19) describes a different behavior due to the higher biogas production because of the fungal treatment, compared with the no pretreated waste. However, the maximum production was lower than that obtained during the *Tv* treatment, for all conditions studied, indicating a reduction in the biogas production due to the fungal growth of *Tv*. In contrast, the treatment with *P. ostreatus* for bigger and smaller particle sizes (figure 20 and 21) showed higher biogas production after 15 d of fungal treatment, and the maximum production was higher than that obtained during the *Tv* treatment for both sizes.

The comparison between the results obtained showed that the biogas production was significantly affected by the different sizes of wheat straw, depending on the fungus used in the treatment. For *T. versicolor* (figure 16 and 17) and *P. ostreatus* (figure 20 and 21) notable differences were not observed, meaning that the reduction in the particle size had a null effect on the anaerobic digestion process. According to Mustafa et al. (2016), milling prior to fungal treatment increases the lignin, hemicellulose and cellulose removal but generally results in lower selectivity, implying that bigger particle sizes can lead to higher biogas production. However, in both particle sizes studied, the biogas production using *P. eryngii* was lower than that using *T. versicolor* and *P. ostreatus.*

To provide a quantitative analysis of the AD process results, the data were fitted using the modified Gompertz equation (equation 3). The parameters obtained are presented in Table 2, which focuses

mainly on the biogas production potential (A) and maximum biogas production rate (μ). The λ parameter was not reported because it was less than 1 d for all the fitted data.

Table 4. Parameters of the modified Gompertz equation (±confidence interval).

Fungus	Decontamination	Size	Pretreatment time [d]	A $[NmL/g_{VS}]$	μ $[1/d]$	R^2
T. versicolor	+	S1	0	292.2 ± 15.5	9.8 ± 1.5	0.9978
			15	292.1 ± 10.4	9.3 ± 2.4	0.9871
			30	310.2 ± 16.7	9.3 ± 2.6	0.9940
		S2	0	266.8 ± 10.2	10.7 ± 1.7	0.9682
			15	244.0 ± 22.3	9.4 ± 1.0	0.9977
			30	282.8 ± 12.0	10.7 ± 2.5	0.9689
P. eryngii	+	S1	0	160.8 ± 15.3	7.8 ± 2.15	0.9855
			15	229.4 ± 14.4	7.6 ± 2.2	0.9820
			30	207.2 ± 13.0	8.2 ± 1.6	0.9967
		S2	0	196.3 ± 22.7	5.7 ± 0.9	0.9942
			15	257.8 ± 16.9	7.3 ± 1.5	0.9813
			30	236.0 ± 19.8	7.4 ± 1.0	0.9824
	-	S1	0	199.7 ± 25.4	9.1 ± 0.2	0.9910
			15	252.3 ± 15.1	5.8 ± 3.5	0.9762
			30	236.2 ± 10.7	6.7 ± 1.2	0.9858
P. ostreatus	+	S1	0	266.5 ± 7.6	10.8 ± 1.2	0.9790
			15	305.8 ± 9.0	10.7 ± 0.8	0.9978
			30	275.8 ± 12.3	13.3 ± 2.1	0.9901
		S2	0	248.6 ± 12.5	13.8 ± 3.4	0.9834
			15	302.4 ± 10.7	12.2 ± 1.8	0.9699
			30	251.7 ± 17.4	14.4 ± 2.2	0.9875
No fungal treatment	+	S1	-	289.2 ± 11.2	16.3 ± 1.6	0.9962
		S2	-	201.8 ± 18.9	12.2 ± 2.0	0.9955
	-	S1	-	260.7 ± 10.6	14.0 ± 3.8	0.9965

(+): decontamination applied

(-): decontamination NOT applied

λ: all values lower than 1.0 d

According to the results obtained during anaerobic digestion process (figures from 16 to 21), a negligible lag time phase existed. This result can be seen clearly in all figures for the different anaerobic digestion treatments because the biogas production started immediately for all curves. As a consequence, the fitted values obtained for λ were equal to zero or less than one d. This phenomenon was attributed to the presence of easily biodegradable compounds in the extract that were most likely present due to the fungal treatment effect and the sterilization processes of the raw material, which were carried out before all experimental processes.

The other parameters calculated (A and μ) were used for comparing the different treatments. According to figure 4, the results obtained after the *T. versicolor* treatment showed no significant differences. This was confirmed from the modified Gompertz parameter analysis, in which no significant differences were present between the maximum biogas production (A) values after 0, 15 and 30 d of fungal treatment using the bigger particle size (S1). The A value obtained for S2 after 0 d of treatment was slightly higher than that after 15 (3 %) and smaller than the value obtained for 30 d (9%); however, the difference was not significant. These parameter values supported the previous results regarding the reducing sugars and enzymatic activities. In both cases, decay was observed after 15 d of fungal treatment for the smaller particle sizes (S2), implying that lower biogas production should be obtained when fungal treatment using a nonselective fungus, such as *T. versicolor*, exceeds 15 d.

In the case of the *P. Eryngii* treatment, no significant differences were obtained between the A parameters under any of the conditions used. Hence, this fungal treatment for enzyme production

did not indicate any negative effects during the anaerobic digestion process but did not indicate improvements either.

When comparing the fungal treatments with applied decontamination, the results indicated that this step did not significantly affect the AD process, although the growth of other microorganisms was observed, which were even more abundant than the white rot fungus studied. According to literature (Akhtar et al. 1998), it has been reported that decontamination might not be always necessary for fungal pretreatment. In contrast, the application of high-temperature decontamination can enhance the biogas production when no fungal pretreatment is used. A value of 289.2 ± 11.2 NmL/gVS for the maximum biogas production (A) using the bigger particle size was obtained when no fungal pretreatment was used but decontamination was applied to the substrate. This value was around 3 % higher than that obtained for the same particle size with no decontamination process applied. Therefore, we concluded that the effect caused by thermal treatment on this lignocellulosic waste was negligible.

Finally, fungal treatment with *P. ostreatus* showed some promising results. As seen previously, high values of reducing sugar release, Lac activity and lignin loss were obtained after 15 d of treatment when the smaller particle size was used (S2). As a consequence, a positive effect was obtained for the AD assay results, in which a higher maximum biogas production (*A*) was obtained after 15 d of treatment for both sizes. A value of 302.4 ± 10.7 NmL/gVS was obtained. While this value was not significantly different from that obtained using *T. versicolor*, combined with the laccase production, we concluded that these treatment conditions are the most promising according to the objective of proposing a biorefinery strategy that includes the production of biogas and high-

value enzymes. In addition, differences in the maximum biogas production rates were observed. The μ parameter after 0 d of pretreatment was slightly higher than that obtained after 15 or 30 d of fungal treatment, but differences were not significant in any case studied

5. CONCLUSIONS

The results obtained in this work showed that it is possible to propose a biorefinery strategy to produce high-value enzymes and biogas to maximize the exploitation of lignocellulosic wastes. However, using different fungus-residue combinations produced different results. Specifically, it was found that fungal treatment on smaller particle sizes (0.5 and 1 mm) of wheat straw with *P. ostreatus* for 15 d could enhance the biogas production while obtaining and maximizing laccase production. A maximum biogas production of 302.4 ± 10.7 NmL/gVS and a maximum biogas rate of 12.2 ± 1.8 NmL/gVSd were obtained. In addition, a maximum laccase activity of 382 U/L was obtained after 8 d of fungal treatment.

As seen in this investigation, the fungus type used is a relevant variable since nonselective fungi, such as *T. versicolor*, can augment the carbohydrate loss, affecting the subsequent biogas production. The treatment time is also a sensitive variable to be considered since fungi, such as *P. ostreatus*, tend to increase the consumption of reducing sugars with longer treatment times. On the other hand, the three fungi used on the study showed laccase and manganese peroxidase activities, but the enzymes appeared during different times for each fungus. While in the case of P. ostreatus, higher laccase activity was obtained during the first 15 d of treatment, manganese peroxidase activity was obtained passed this time.

Another significant variable studied in this work, due to its expensive cost, was decontamination. While it did not greatly affect the biogas production, its effect on the fungal treatment efficiency must be considered.

RECOMMENDATIONS

Further investigations should focus on finding new ways to improve the results obtained in this work.

Based on the results obtained in this work, a statistical analysis could be proposed. A factorial design with discrete values based on this study on the overall process could be evaluated.

The evaluation of the decontamination process during fungal treatment must be evaluated in order to verify the effectiveness of this treatment and its real benefits for the enzyme production for each fungi of the study.

On the other hand, the purification and extraction of the enzymes must be studied. Depending on how invasive the process could be for the overall sequential proposed strategy, its effect on the fungus or on the AD process should be taken into consideration. Furthermore, purification and extraction of enzymes is usually expensive, and therefore, an economical evaluation should be addressed in order to make the proposed strategy viable from an economic point of view.

Finally, different strategies to increase the enzymes productions must be assessed such as adding inducers likeCu^{+2}, with the objective of increasing laccase production.

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APENDIX A

SCIENTIFIC PRODUCTION

Different scientific communications have been realized during the construction of this Thesis.

2016 Poster Communication

XII Latin American Workshop and Symposium on Anaerobic Digestion

October 23th to 27th, Cuzco, Peru

"Study of a biological pretreatment using *Trametes versicolor* on wheat straw for enhancing biogas production"

Authors: S. Albornoz, C. Palma, A. Carvajal

2015 Poster Communication

14th World Congress on Anaerobic Digestion

November 15th to 18th, 2015, Viña del Mar, Chile

"Study of a biological/thermal pre-treatment to improve anaerobic digestion of wheat straw"

Authors: S. Albornoz, D. Vasquez, C. Palma, A. Carvajal.

2017 Paper (awaiting for approval)

"A strategy for combined enzyme and biogas production from wheat straw in a biorefinery"

Authors: Sandra Albornoz, Carolyn Palma, Andrea Carvajal(*)

Submitted to Biomass & Bioenergy Journal, waiting for evaluation.