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LIGNOCELLULOSIC WASTE VALORISATION STRATEGY THROUGH ENZYME AND BIOGAS PRODUCTIO

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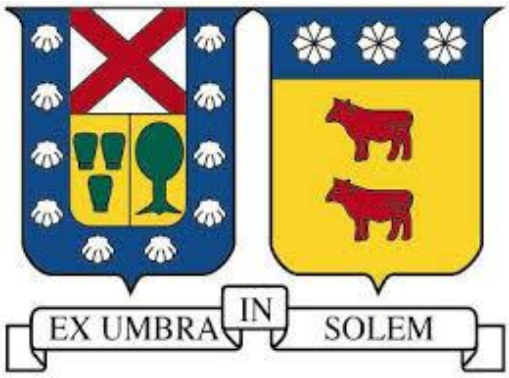

UNIVERSIDAD TÉCNICA FEDERICO SANTA MARÍA

DEPARTAMENTO DE INGENIERÍA QUÍMICA Y AMBIENTAL

LIGNOCELLULOSIC WASTE VALORISATION STRATEGY THROUGH ENZYME AND BIOGAS PRODUCTION

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Dedicated to Carolina Jara Lagos

Don’t let the world change your smile

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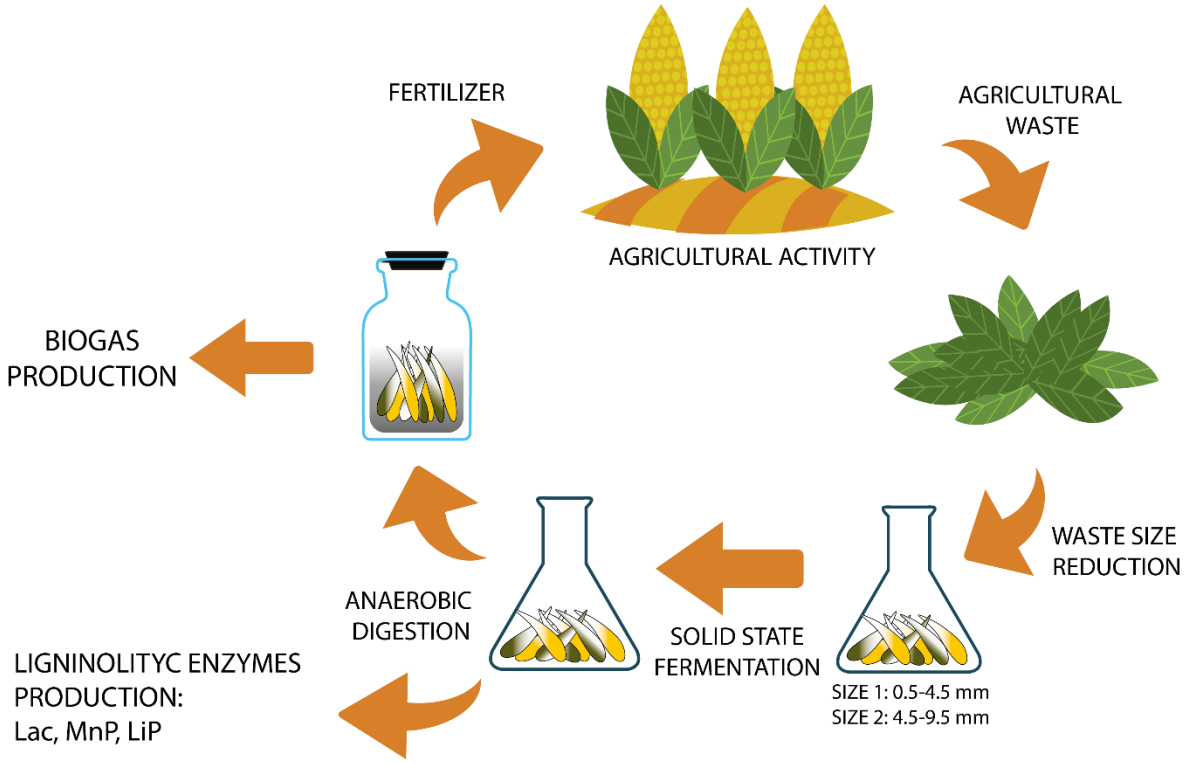
GRAPHICAL ABSTRACT



RESUMEN

Los residuos lignocelulósicos son, por lo general, pre-tratados para facilitar la etapa de hidrólisis durante el proceso de digestión anaerobia. Se evaluó un proceso secuencial consistente en la fermentación de sustrato sólido, realizada por hongos de putrefacción blanca, y digestión anaerobia en rastrojo de maíz para producir enzimas ligninolíticas y biogás. La producción de enzimas fue cuantificada cada 3 días durante un mes a 30 °C, y se comparó en tres cepas de hongo y dos tamaños de partículas del residuo. Dentro de los principales resultados, se obtuvo una mayor actividad enzimática de lacasa con la cepa *Pleurotus eryngii* en comparación con las cepas *Pleurotus ostreatus* y *Trametes versicolor*. Además, esta actividad mejoró en un 16% cuando se utilizó cobre como inductor enzimático. Por otro lado, la mayoría de las condiciones estudiadas mostraron una disminución en la producción máxima de biogás comparada con el residuo sin tratar. La adición de cobre disminuyó la producción de biogás en un 20%. A pesar de lo anterior, *Pleurotus eryngii* mostró resultados prometedores que permitieron un aumento del 19% en la producción de biogás y altos valores de producción de enzimas.

ABSTRACT

Lignocellulosic wastes are generally pre-treated to facilitate the hydrolysis stage during the anaerobic digestion process. A sequential process consisting of solid substrate fermentation carried out by white rot fungi and anaerobic digestion was evaluated on corn stover to produce ligninolytic enzymes and biogas. The enzyme production was quantified every 3d for a month at 30°C, and three fungal strains and two particle sizes of waste were compared. Of the main outcomes, *Pleurotus eryngii* produced the highest laccase enzyme activity compared with *Pleurotus ostreatus* and *Trametes versicolor*. Furthermore, this activity was improved by 16% when copper was used as an enzyme inducer. On the other hand, most of the conditions studied showed a decrease in maximum biogas production compared with untreated waste, the addition of copper decreased biogas production by 20%. Despite the above, *Pleurotus eryngii* showed promising results allowing a 19% increase of biogas production and high enzyme production values.

1. INTRODUCTION

Currently in the world, more than half of the cultivated area are used for crops because they are considered the most important food source for human consumption. In addition, the primary cereals in the human diet are maize, rice and wheat, which represent 42.5 % (FAO, 2016).

Corn stover is a by-product generated by corn grain cultivation, and their production is estimated to be in a ratio of 1:1. Corn stover includes the husks, cobs, leaves and stalks. In addition, it is considered a lignocellulosic waste (LW) and typically comprises 37.5 % cellulose, 22.4 % hemicellulose and 17.6 % lignin (Mosier et al., 2005). Currently, the cellulose and hemicellulose portion can be converted to ethanol, and the lignin portion can be burned as a boiler fuel for electricity/steam generation. However, ethanol production presents several limitations regarding its resource efficiency. Thus, biogas production by anaerobic digestion appears as an attractive alternative for corn stover management because the process is considered better with respect to energy efficiency, life-cycle emissions and biomass conversion (Börjesson and Mattiasson, 2008).

Lignocellulosic biomass is a complex solid substrate matrix that limits the hydrolysis stage on anaerobic digestion (AD), generating a bottle-neck for the next stages (Rouches et al., 2016). Several pre-treatment methods are used to facilitate the hydrolysis stage, maximizing substrate accessibility (Chandra et al., 2007). Pre-treatments are a combination of various types of physical-chemical techniques applied before the AD process, and they are used to obtain improved

yields (Carrere et al., 2016). The pre-treatment process is classified into different categories such as biological, chemical, physical and a combination of these (Chandra et al., 2007).

Biological pre-treatments are considered a low-cost alternative, and they are typically used to treat lignocellulosic biomass because the fungal metabolism can modify its chemical composition (Ravindran and Jaiswal, 2015; Rouches et al., 2016). Generally, soft and brown rot fungi primarily degrade the hemicellulose while imparting minor modifications to lignin (Chandra et al., 2007). White rot fungi (WRF) are microorganisms that can degrade lignin through enzyme production. In the literature, it is noted that the application of WRF has been studied with greater emphasis on ethanol production than the anaerobic digestion process (Rouches et al., 2016). The major oxidative ligninolytic system comprises three main enzymes: lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac) (Ravindran and Jaiswal, 2015).

Lac is a multi-copper enzyme that can oxidize phenols and aromatic amines (Rodríguez and Toca-herrera, 2007), making it an attractive and demanded component because it has many applications in the bioremediation, food, textile, nanobiotechnology, cosmetics and synthetic chemistry industries (Couto et al., 2006). To increase Lac secretion, the effects of the addition of inducers such as xylidine, ferulic acid, veratryl alcohol, pyrogallol and copper have been studied (Vrsanska et al., 2016). In addition, previous studies have shown that copper is an efficient inducer when dosed as copper sulphate ($CuSO_4$) to increase Lac production (Fillat et al., 2015).

On the other hand, a physical pre-treatment approach used for the AD process is comminution where the particle size of the substrate is reduced, causing an increase in the hydrolysis stage rate. Indeed, particle size reduction has increased biogas production, especially in the case of substrates with a high content of slowly biodegradable materials (Jędrczak and Królik, 2007) because of their influence in the initial degradation rate (Raposo et al., 2012).

According to the literature, it is possible to find studies about biological pre-treatment application by WRF under aerobic conditions on LW to improve the AD process. Specifically, lignocellulosic substrates as wheat straw, rice straw, corn stalk, and wood fibre have been subjected to biological treatment with WRF to enhancement methane or biogas production on AD process (Carrere et al., 2016; Rouches et al., 2016). Also, forest residues as hazel and acacia branches have been subjected to fungal pre-treatment by *Ceriporiopsis bubvermispora* causing a significant increase on its biomethane potential (Liu et al., 2017). In addition, the study of combined pre-treatments as fungal and size reduction by milling on rice straw has shown the significant effect on lignin, cellulose, and hemicellulose degradation, causing the methane yield increase during anaerobic digestion (Mustafa et al., 2017). Also, the improvement of lignin degradation during bagasse hydrolysis process for biofuels production, by dossing supplements on fungal treatment (Mishra et al., 2017). On the other hand, ligninolytic enzyme production such as Lac, MnP and LiP, has been reported also in several studies using different supports as wheat straw, grape, banana waste and corn (Rodríguez Couto and Sanromán, 2005). However, the evaluation of enzyme and biogas production in a single process has not been reported to date.

2. OBJECTIVES

According to the background described above, this study proposes to evaluate the feasibility of producing enzymes and biogas from a single lignocellulosic waste -corn stover- through the sequential application of a physical-biological treatment followed by an anaerobic digestion process.

The global process was studied for two particle sizes of lignocellulosic waste, and the response against three different strains of WRF were compared.

Finally, the copper inducer presence was evaluated in the production of both stages, enzyme and biogas.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Waste conditioning

Corn stover was the lignocellulosic waste used in this research project. It was collected in a single day from a rural vegetable street market in Santiago, Chile. The corn stover was dried at room temperature until achieving 10 % moisture. Next, it was manually chopped and subsequently mechanically minced. Later, the material was sieved and collected in two fraction sizes (Gilson Company Inc); the small size S1 was between 0.5 and 4.5 mm, and the large size S2 was between 4.5 and 9.0 mm.

The waste characterization was performed through the quantification of total lignin in the biomass (TL) using the NREL method (Sluiter et al., 2012), total Kjeldahl nitrogen (TKN) using acid digestion and distillation (AOAC, 2000), and total solids (TS) and volatile solids (VS) using standard methods (APHA), 2005).

3.1.2. Fungi production

Three strains of WRF were used in this study: *Pleurotus eryngii (Pe), Pleurotus ostreatus (Po)* and *Trametes versicolor (Tv)*. These fungi were donated by the Biological Research Center (*CIB*) in Madrid. The strains were maintained at 4 ºC in slants; once a year, all tubes were renewed.

Fungus proliferation was carried out using agar slants in which a small portion of an individual strain was inoculated on MEA plates. After 7 d of incubation, the actively growing mycelium covered all plates; next, 3 plugs of approximately 1

cm^2 from a MEA plate were transferred to a 250-mL flask for submerged culture using 100 mL of liquid growth medium (Salvachúa et al., 2011). The fungus pellets were obtained after 10 d at 200 rpm and 30 °C or until the reducing sugar concentration was reduced by 90 %.

3.2. Experimental methods

This study exhibited the effect caused by three main variables: two waste sizes (S1 and S2), three fungal strains (*Pe*, *Po* and *T*v) and copper dose as laccase inducer (Cu+2). On the other hand, there are two main studied responses, enzymes and biogas production. Therefore, the experimental procedure is described as the enzyme production stage and biogas production stage. For both experiment types, every condition was performed in triplicate, allowing statistical comparison among different groups of data. Finally, to reduce the number of trials, the copper effect was only evaluated for S1 with the *Pe* and *Po* strains.

Table 1: Experiments performed with different experimental conditions

SIZE	**CU^{+2}**	**WHITE ROT FUNGI STRAIN**		
		Pe	*Po*	*Tv*
S1	no	X	X	X
	yes	X	X	--
S2	no	X	X	X

3.2.1. Enzyme production

The enzyme production stage was performed by dosing 3 g of conditioned waste and 5 mL of distilled water in 250-mL flasks, sealed with cotton stoppers. All

samples were sterilized using a thermal treatment at 121 °C 30 min prior to biological treatment to avoid the growth of other microorganisms (Carrere et al., 2016). After cooling, 6 mL of fungus pellets and 3 mL of sterilized water were added to the flasks and manually mixed. Waste SSF was performed in an incubator (VELP-FOC 215E) at 30 °C statically (Sindhu et al., 2016) and ~82 % moisture. For the inducer trials, the sterilized water dosage in the previous sterilization was a $CuSO4{\cdot}5H2O$ solution. The final concentration of copper inducer in each trial was 1.76 mg/g. This concentration was identified as sufficient to increase laccase production under solid state fermentation conditions in previous experiments (Rodríguez and Toca-herrera, 2007).

The monitoring of the biological treatment was made as a function of time; hence, 3 flasks were removed every 3 d for 30 d (destructive assays) and were mainly characterized in terms of enzymatic activity, carbohydrate, reducing sugars and volatile fatty acid contents; all these analyses were carried out in a prepared liquid extract. Furthermore, at 0, 15 and 30 d, three extra flasks per sample were taken for the exclusive measurement of TS, VS, TKN and TL; in these cases, the analyses were carried out in the solid samples.

The liquid extract was prepared adding 50 mL of distilled water, and then the content of the flask was manually homogenized and later stirred at 250 rpm and 10 °C for 60 min. The resulting pulp was centrifuged for 10 min at 5000 rpm and 10 °C, and the collected supernatant was used for the corresponding analyses. For carbohydrate and volatile fatty acid analysis, the supernatant was previously filtered using a 0.22-µm pore size.

The quantified ligninolytic enzymes were Lac, MnP and LiP. First, the enzymatic activity of Lac was measured by monitoring the oxidation of ABTS (2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt, ≥98 % (Sigma Aldrich) in acetate buffer (pH 5.0) at 436 nm, using an extinction coefficient of 29,300 M-1cm-1 (Muñoz et al., 1997). Second, the MnP activity was quantified by monitoring the oxidation of DMP (2,6-dimethoxyphenol, 99 %; Sigma Aldrich) in sodium tartrate (pH 5.0) at 469 nm, using an extinction coefficient of 27,500 M-1cm-1 (Martinez et al., 1996). Third, LiP activity was determinated by the oxidation of veratryl alcohol (3,4-dimethoxybenzyl alcohol, 96 %; Sigma Aldrich) in sodium tartrate (pH 3.0) at 310 nm, using a molar extinction coefficient equal to 9,300 M-1cm-1 (Tien and Kirk, 1988). The amount produced by each enzyme was quantified as enzyme units (U), corresponding to the amount of enzyme that catalyses the reaction of 1 µmol of substrate per min. The enzymatic activity was expressed as enzyme units per litre of extract (U/L).

The reducing sugar content was quantified using 3.5-dinitrosalicylic acid (Sigma Aldrich) and the DNS method (Miller, 1959). The calibration standard used was glucose (D-(+)-glucose, ≥99.5 %; Sigma Aldrich). The analyses were performed using a SPECORD 210 plus spectrophotometer. In addition, specific carbohydrates (CH) (glucose, fructose and galactose) and volatile fatty acids (acetic, propionic and butyric) were quantified by HPLC (Agilent Technologies Model Infinity 1260). The method was carried out using water as the eluent phase and an HPX-87 column and micro-guard cation H Refill cartridge pH 1-3 precolumn (Aminex) at 45 ºC (De Sá et al., 2011).

3.2.2. Biogas production

According to the protocol of the biomethane potential (BMP) (Angelidaki et al., 2009), the AD process was evaluated for 40 d in batch systems. After the enzyme production stage -specifically at 0, 15 and 30 d of biological treatment- samples (assays performed in parallel to enzymes production stage) from fermented waste were moved to 250-mL Pyrex bottles. Anaerobic inoculum from a waste water treatment plant of a Chilean brewery company, micronutrients and a solution of $NaHCO_3$ as buffer were added (Holliger et al., 2016). The assays were performed using a substrate/inoculum volatile solid ratio of 1 (Raposo et al., 2006). The air content in the bottles was displaced by nitrogen.

Biogas production contained in the bottle was indirectly quantified by daily measurement of the temperature and pressure using an IFM electronic pressure transducer. The biogas composition was quantified by gas chromatography with a thermal conductivity detector (Agilent Technologies Model 7890-B). The method consisted of a system with two capillary columns working in parallel (Molsieve 5A and PoraBOND Q) to enable the measurement of CH_4, CO_2, O_2, N_2 and H_2S. The carrier gas used was helium, and the oven temperature was 42 ºC. Additionally, calibration was performed using an AIRGAS Standard with a composition of 28 % CO_2, 2 % H_2S and CH_4.

3.2.3 Quantitative analysis

Accumulated biogas production obtained in BMP experiments were adjusted using the Transfer Function (TF) (Parra-orobio et al., 2017), as presented in equation 1.

$$V\ (\mathrm{t}) = \mathrm{P}_{max}\left(1 - \exp\left(\frac{-R_{max}(t-\lambda)}{\mathrm{P}_{max}}\right)\right) \quad \text{(equation 1)}$$

Equation 1 relates the following variables: V as the accumulated biogas production [mL/g_{VS}]; P_{max} as the maximum biogas production [mL/g_{VS}]; R_{max} as the maximum biogas production rate [mL/g_{VS}/d]; and λ as the lag phase period [d]. These parameters were determined by applying the non-linear regression approach using SigmaPlot™ version 11, Systat Software. All results obtained were presented as average values and standard deviations.

4. RESULTS AND DISCUSSION

4.1 Waste Characterization

The characterization of the conditioned material showed that the corn stover contents of TS and VS were 920.6 (±0.89) g/kg and 890.6 (±0.07) g/kg, respectively. According to TKN analysis, the waste had sufficient organic nitrogen content for fungi growth. The concentration of reducing sugars in the extract was similar for both particle sizes, 6.82 (±0.24) g/L for S1 and 6.51 (±0.35) g/L for S2. The contents of organic nitrogen for S1 and S2 were 6.60 (±0.09) and 6.98 (±0.33) mgN/g, respectively. This characterization is a significant factor for the evaluation of waste selection because a low nitrogen content could promote lignin degradation by WRF, while a high content can inhibit it (Rouches et al., 2016) The TL contents were 24.27 (±0.90) % and 18.43 (±1.10) % for S1 and S2, respectively.

Furthermore, the difference between both samples was identified as significant by the comparison of the confidence intervals of both means. The slight increase in TL contents for S1 to S2 could be explained by the mechanical properties of the fibre when it is subjected to grinding. From this point of view, the components of the waste would have a different response to the treatment due to their hardness or fragility, modifying the composition of the treated compound. This behaviour has been previously observed in the treatment of sunflower oil cake, in which the degradation efficiency was higher for larger particle sizes due to a lower fibre content (De la Rubia et al., 2011).

4.2.1. Enzyme production

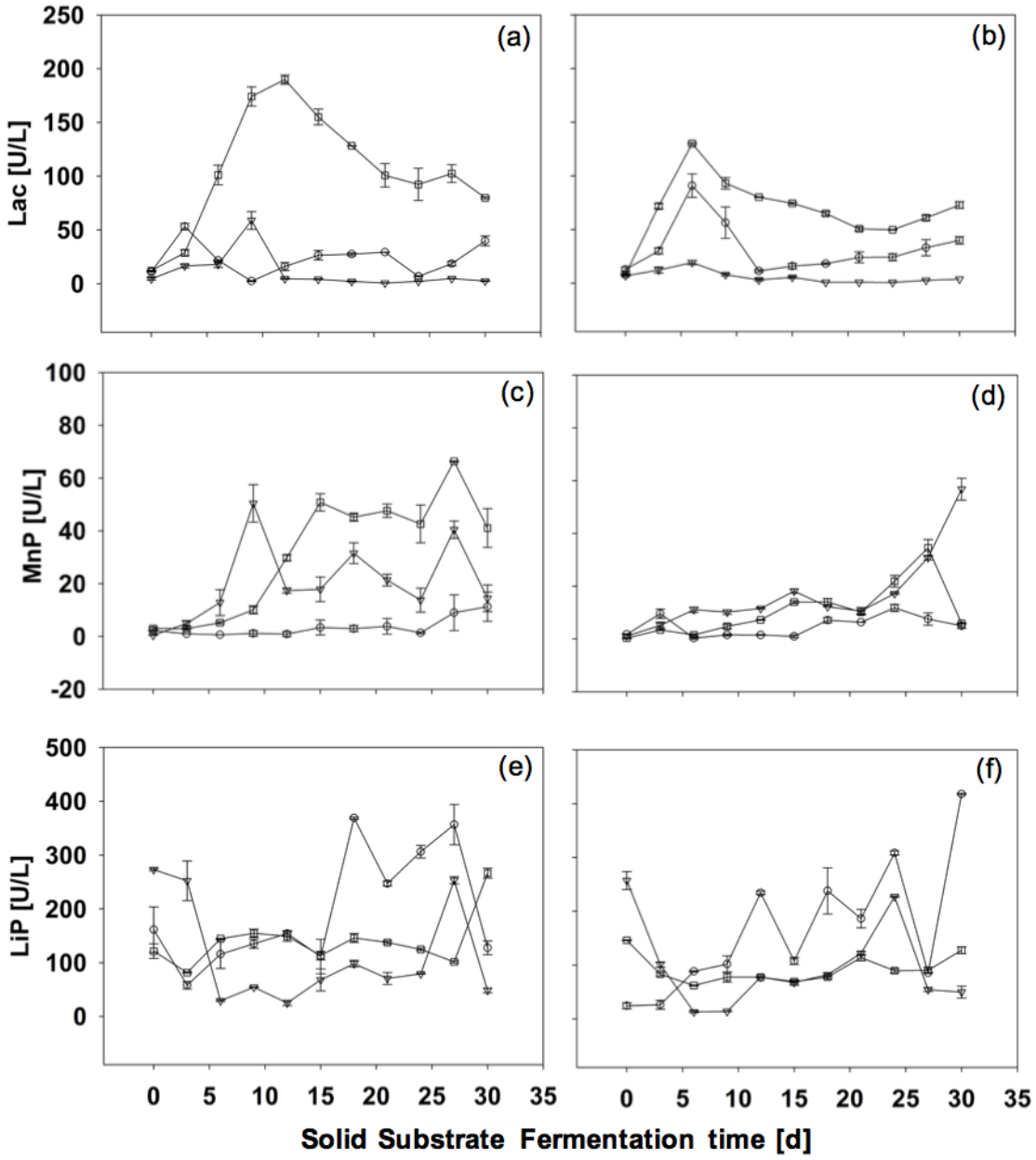


Figure 1: Enzymatic activity of Lac (a) S1 and (b) S2, MnP (c) S1 and (d) S2, LiP (e) S1 and (f) S2 (symbols: diamond, *Tv*; circle, *Po*; triangle, *Pe*).

The first objective of this study was to evaluate the feasibility of enzyme production from agricultural waste (AW), but information regarding which enzymes are produced or at what specific time fungal fermentation occurs is not available in the literature for this waste. The results presented in Figure 1 shows

the enzymatic activity curves for each studied case. The laccase activity obtained (Figure 1.a and 1.b) was different according to the waste size, being greater for S1 than for S2 for *Pe* and *Po* strains. In particular, the maximum Lac activity was obtained for the *Pe* strain at 12 days of fungal treatment for small particle sizes, reaching a peak of 189.7 (±4.4) U/L in the extract. By contrast, for *Tv* fermentation, the maximum Lac production was reached for S2 at 6 d. Clearly, these first results indicated different behaviour responses caused by the interaction of different fungi with one specific waste. Other studies of enzyme production from low-cost sources such as wheat straw with *Trametes versicolor* during 28 d of fungal fermentation reached a peak of Lac activity of ~120 U/L after 7 d of treatment (Dinis et al., 2009). Thus, a response was obtained for Lac production from corn stover, when the fermentation condition used was a small waste size and the *Pe* strain represented a good alternative, compared with the other experimental conditions and with other substrates studied in the literature.

The MnP activity (Figure 1.c and 1.d) also recorded higher values for S1 than for S2. The maximum production was obtained first for *Po* at 9 days of treatment with 50.4 (±7.1) U/L and then for *Pe* with a peak of 66.4 (±0.17) U/L at 27 days of treatment, both at a small particle size. On the other hand, for tests using a large particle size, the highest MnP activity was obtained for *Po* over the period of pre-treatment but after 25 d of fungal treatment. Finally, it is important to indicate that MnP activity of *Tv* strain was detected during the assays; however, the values reached were close to zero for both particle sizes. This behaviour observed was like that in other trials reported for treatment using wheat straw, in which the values of MnP activity with *Trametes versicolor* were lower than those with the

Bjerkandera adusta and *Phlebia rufa* strains and similar to those with the *Ganoderma applanatum* strain (Dinis et al., 2009).

Finally, the maximum production of LiP at S1 (Figure 1.e) was obtained for *Tv*, reaching a value of 369.0 (±1.8) U/L at 18 days of treatment. The peak of LiP activity at S2 (Figure 1.f) was reached by *Tv* at the end of the trial period with a value of 418.06 (±1.22) U/L. In this case, the values of activity both for S1 and S2 were similar, but were slightly higher for S2. Other authors did not report the LiP activity on wheat straw with *Tv* for 28 d of biological treatment (Dinis et al., 2009). The multiple and close peaks detected in the enzymatic activity (Figure 1) could be attributed to the action of proteases produced by WRF. According to the literature referent to enzymes activities determined under SSF conditions, the decay of extracellular enzymes activities is generally caused by protease-mediated degradation. A hypothesis mentioned in previous researches indicated that this process occurs because the proteases break down proteins (secreted and released) into the medium to recycle nitrogen (Dosoretz et al., 1990). On the other hand, the ligninolytic enzymes production periods have been reported after limitation of nutrients, specially carbon and nitrogen (Akpinar and Urek, 2012). From this point of view, the multiple peaks detected could be a representation of the enzyme production variation in function of a sequential action of enzymes.

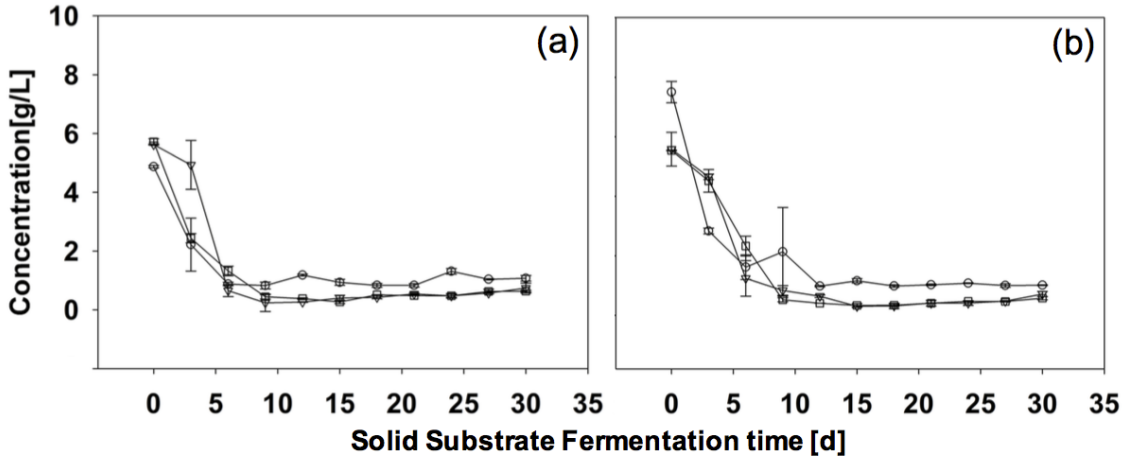


Figure 2: Reducing sugar content of (a) S1 and (b) S2 (symbols: diamond, *Tv*; circle, *Po*; triangle, *Pe*).

For a more detailed analysis, it is necessary to indicate that the activity of both enzymes, Lac and MnP, reached their highest value from the 6th d of biological treatment, coinciding in time with a significant reduction of the reducing sugar content in the extract (Figure 2.a and 2.b). These results suggested that the high levels of soluble sugars at the beginning of the culture promoted fungus growth and, consequently, generated the corresponding lag phase in enzyme production until inhibition by the substrate. This effect had been previously indicated for wood fermentation by WRF for laccase production, in which the start of enzyme production coincides with the time close to complete consumption of glucose (Galhaup et al., 2002). In all cases, after 9 d of fungal treatment, the reducing sugar concentration remained constant over time. Finally, it would be possible to indicate that the consumption of sugars at the beginning of the assays could be attributed to fungus growth, but the different nutrient requirements of the three strains used provoked a different response to enzyme secretion.

A more detailed analysis of the CH composition in the extract reinforced previous results of reducing sugar consumption. The results obtained showed a decrease in the presence of glucose, sucrose and fructose over time, with glucose being the fastest sugar consumed and fructose being the slowest. More specifically, for small particle sizes the initial concentration of glucose, sucrose and fructose detected were 7.05 (±0.71) mM, 2.41 (±0.30) mM and 9.53 (±0.56); and for large particle sizes, the glucose detected was 4.98 (±0.32) mM. After 3, 6 and 9 d of biological treatment, no glucose was detected for the *Tv*, *Po* and *Pe* strains, for small particle sizes. For greater waste sizes, the tendency was similar, except with the *Po* strain, where the consumption of glucose was not complete over time. The CH degradation time could explain the Lac activity values obtained by the strains due to its faster consumption restricting fungus growth. The higher values of Lac activity were reached by *Pe*, which presented the slowest CH consumption rate. By contrast, the lowest values of Lac activity were reached by the *Tv* strain, which presented the faster CH degradation rate. In addition, the quantification of VFA reported the concentration of acetic and propionic acids until 9 d of biological treatment; thereafter, it was not possible to detect the compounds.

Thus, the difference observed for enzymatic activities between small and large particle sizes was significant and could be attributed to the initial total lignin content in the conditioned waste and/or that the small particle size favoured the fungal penetration into biomass respect to the large particle size (Sindhu et al., 2016). From all previous analyses, it can be concluded that Lac and MnP activities were more sensitive to particle size than LiP, and both enzymes could be produced with a lower fermentation time than 10 d.

This phenomenon observed can be explained by LW composition and fungal growth mechanism. Several studies have indicated that when *Tv* (Johansson et al., 1993) and *Po* (Becker and Sinitsyn, 1993) are cultivated on defined medium can produce Lac, MnP and LiP, while *Pe* (Muñoz et al., 1997) produces only Lac and MnP. That is to say, the enzymatic activity measured for different process depends of several factor as fungal specificity and nutrients sources provided in limitation and/or sufficiency condition. In addition, corn stover has a content of 0.98 g/kg of Mn^{+2} (Mullen et al., 2010), which can operate as a natural inductor of manganese peroxidase, favouring its stable activity respect to LiP. Fungal growth is sensitive to many factors as inhibitors, biomass type, pH, moisture, incubation temperature, incubation time, aeration (Sindhu et al., 2016). Even, several inhibitor compounds presence in the LW or as a product of the fungal treatment process could affect the enzymatic behaviour detected on this study (Johannes and Majcherczyk, 2000)

4.2.2. Enzyme production in the presence of inducer

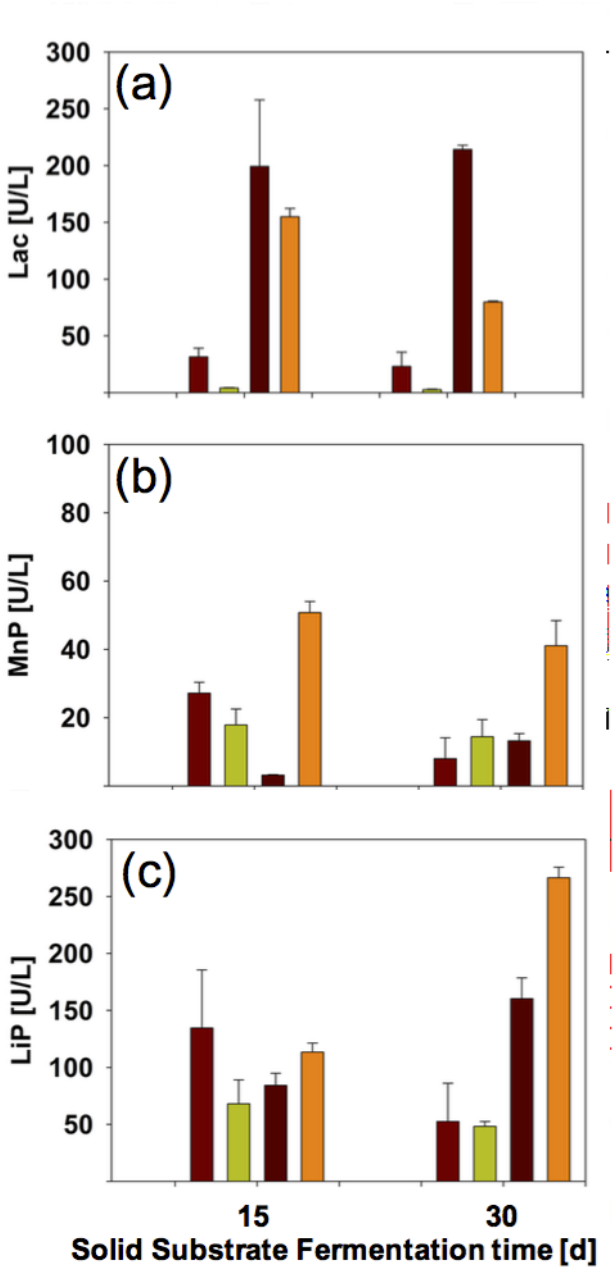


Figure 3: Enzyme activity of (a) La, (b) MnP, and (c) LiP for experiments with Cu^{+2} inducer dosing (colours: red, *Po* with cu^{+2}; green, *Po* without Cu^{+2}; dark brown, *Pe* with Cu^{+2}; orange, *Pe* without Cu^{+2}).

Previous results indicated that the most produced enzyme in the shortest time was laccase *(4.2.1. section)*; thus, the opportunity to increase this maximum value by adding a specific inducer should be considered. In fact, the comparative effect of enzyme activity due to the inducer addition showed an increase in Lac

activity for both fungi used (Figure 3.a), in which the peak detected at 15 d of treatment was 7.7 times higher for *Po* and 1.29 times higher for *Pe*. Although the increase in Lac activity was more significant in *Po* culture than in *Pe* culture, the maximum value reached was higher for *Pe* than for *Po*, 5 times higher at 15 d of fungal treatment, a finding that coincided with lower values of TL loss with respect to Po without inducers (Table 2). On the other hand, MnP and LiP activities (Figure 3.b and 3.c) decreased for *Pe* fungal treatment by the presence of the Lac inducer, indicating that the fungus promoted the secretion of the induced enzyme, with respect to the non-induced ones. However, the behaviour observed of MnP and LiP enzymes for *Po* was not equal.

Table 2: Weight and total lignin (TL) loss of corn stover treated with WRF under solid state fermentation.

Strain-Size-Inducer	Weight Loss [%]		TL Loss [%]	
	15 [d]	30 [d]	15 [d]	30 [d]
Po-S1	14.7 ± 0.2	24.9 ± 0.2	19.6 ± 0.7	34.2 ± 0.6
Po-S2	7.1 ± 0.00	21.0 ± 0.5	15.0 ± 0.7	31.4 ± 0.9
Pe-S1	9.6 ± 0.1	16.2 ± 0.1	5.5 ± 0.4	14.0 ± 0.7
Pe-S2	7.1 ± 0.2	12.1 ± 0.1	25.2 ± 0.7	26.1 ± 0.5
Tv-S1	20.3 ± 0.4	30.3 ± 0.5	24.7 ± 0.8	30.3 ± 0.9
Tv-S2	20.4 ± 0.2	28.8 ± 0.1	14.7 ± 0.6	27.0 ± 0.7
Pe-S1-Cu	13.7 ± 0.6	18.5 ± 0.5	13.3 ± 0.3	13.7 ± 0.4
Po-S1-Cu	13.6 ± 0.9	18.1 ± 0.3	5.4 ± 0.3	8.3 ± 0.3

On the other hand, the weight and TL loss values of AW were quantified for 15 d and 30 d of biological treatment and were compared with the values obtained without the treatment (0 d) (Table 2). The higher weight loss was reported by the *Tv* strain for both particle sizes, reaching values of nearly 30 % for S1 and 28 % for S2. By contrast, the lowest value obtained for S1 was 7 %, and it was observed

for both *Pe* and *Po* strains; however, for S2, the lowest value was 12 %, obtained with Pe. These data could indicate the growing rate of the strains in the following order: *Tv*> *Po*> *Pe*.

The variation of corn stover degradation with different strains of WRF had been reported previously after 30 d of fungal treatment at 28 ºC and ~ 74 % moisture level, the range of weight loss obtained being between 9.8 ± 0.1 % and 52.8 ± 1.3 % using *Coprinus sp.* and *Phanerochaete chrysosporium*, respectively (Saha et al., 2016).

In this study, the assays in the presence of the inducer showed different responses for each strain. In *Pe* assays, the corn stover degradation was increased with respect to the experiment without the inducer dose; however, in the *Po* assays, the behaviour was the inverse. According to the TL loss values, the higher lignin degradation for S1 and S2 was reached with the *Po* strain. Overall, the lowest TL loss at the end of the treatment period was obtained for S1 corn stover treated with *Pe* and *Po* strains and the inducer dose. In addition, the weight loss was always higher for small particle sizes than for large particle sizes. However, the response of TL loss was inverse. Finally, higher values of Lac activity were reported before 15 d of biological treatment, coinciding with the lower values of lignin losses. The higher Lac peak obtained without inducer occurred after 12 d of biological treatment of S1 with the *Pe* strain, with a TL loss of 5.5 (±0.4) %, measured at 15 d. However, the data suggested that MnP activity increased over time of biological treatment. The same situation was observed in the treatment of wheat straw with four strains of WRF, where the peak of Lac

activity was obtained at 7 d, and the MnP peaks were detected at the end of the period of treatment (28 d) (Dinis et al., 2009).

4.3. Biogas production

The second objective of this study was to evaluate the feasibility of producing biogas from LW after biological treatment. Particularly, it was required to evaluate if this sequential process can improve or inhibit the second stage. Addressing this issue was not trivial because the enzymatic expression and effects on the waste were different depending on the fungus applied. Therefore, to identify kinetics parameters and differentiate the effects on the AD process, the results were adjusted to the Transfer Function (Table 3) and the calculation base used for all samples was the VS content in the raw LW.

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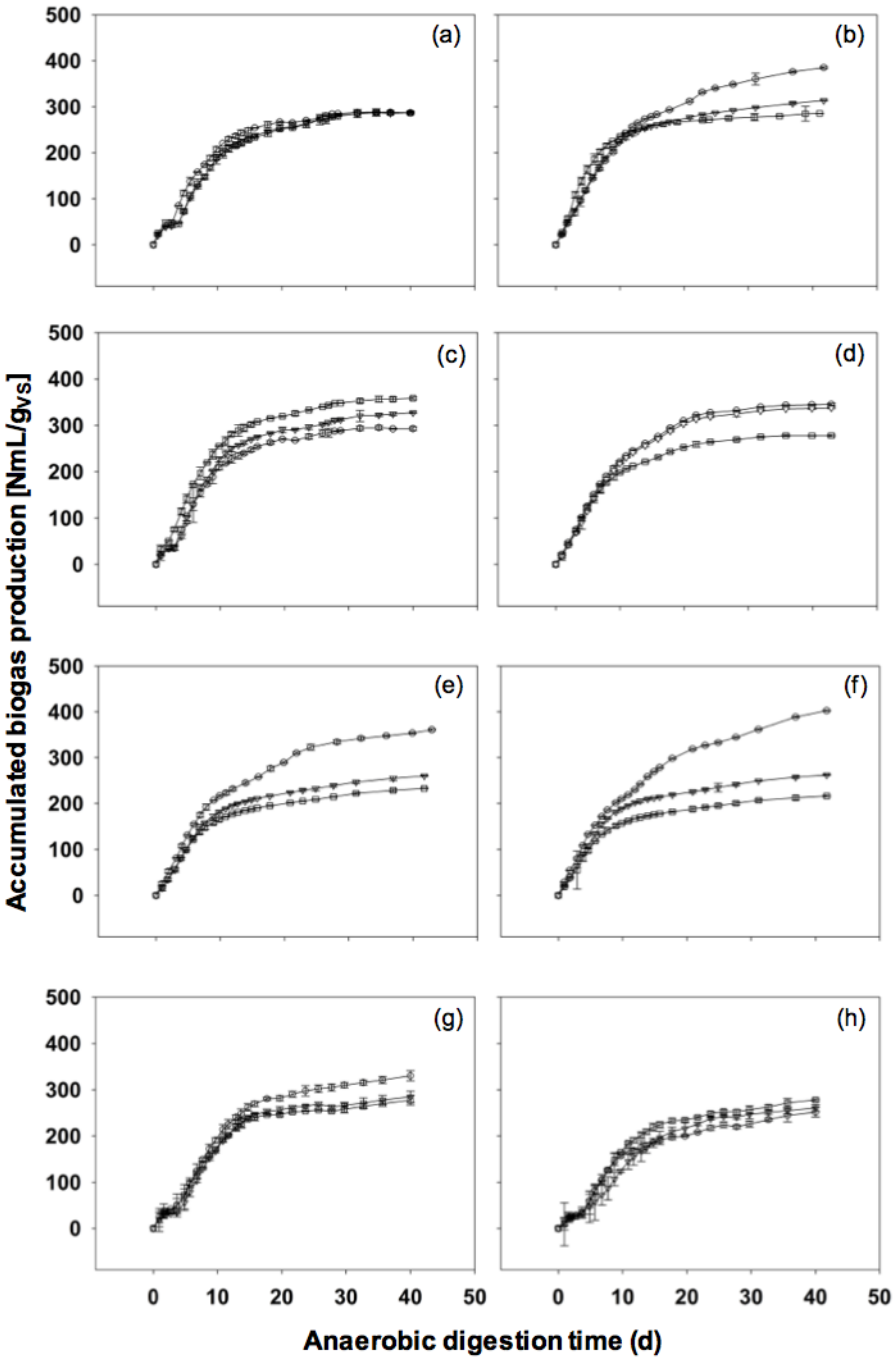

Figure 4: Accumulated biogas yield of corn stover after 0, 15 and 30 d of biological treatment with *Po* (a) S1 and (b) S2, *Pe* (c) S1 and (d) S2, *Tv* (e) S1 and (f) S2 and assays with enzyme inducer (g) *Po* and (h) *Pe*. (Symbols: circle, 0 d; triangle, 15 d; square, 30 d).

Figure 4 shows the biogas production curves obtained for each condition. Because of the fungal treatment, the biogas production presented different

behaviours with each pre-treated waste, depending on the strain. The treatment by *Po* showed no significant effect on biogas production for S1 (Figure 4.a); however, for S2 (Figure 4.b), the biogas production decreased with the increase in fungal treatment time. By contrast, biogas production assays after 30 d of *Pe* treatment with the S1 size showed an increase of 19 % compared with raw waste (Figure 4.c). On the other hand, the result obtained at the S2 size was completely different (Figure 4.d), where 30 d of biological treatment were unfavourable, causing a decrease in biogas production by 12.5 %. Finally, the biological treatment using *Tv* caused a decrease in biogas production for both sizes studied (Figure 4.e and 4.f). It should be highlighted that these biogas production yields were quantified using the initial VS content in the raw material. In this context, when the VS content reduction by fungal treatment was considered the methane yield production changed significantly (*Supplementary Material data of this work can be found at the end of the document*). Specifically, the maximum methane production yield reached after 30 d of fungal treatment with *Pe* showed an increase in 55.8 % (362.7 NmL/g_{VS}) for S1 and for S2 showed an increase in 8.39 % (344.37 NmL/g_{VS}), both compared with raw waste. All these results are consistent with the literature in which several studies have reported the improvement of the anaerobic digestion process due to the application of biological pre-treatments (Mustafa et al., 2017, 2016)

Table 3: Parameters of the transfer function model for corn stover after fungal treatment.

Strain-Size-Inducer	Parameter	Biological treatment time [d]		
		0	15	30
***Pe*-S1**	P_{max}, mL/g_{VS}	301.56 ± 4.85	331.35 ± 7.14	360.49 ± 3.55
	R_{max}, mL/g_{VS}/d	34.58 ± 1.49	36.44 ± 2.00	43.48 ± 1.26
	λ, d	0.75 ± 0.22	0.62 ± 0.17	0.30 ± 0.11
	R^2	0.9877	0.9788	0.994
***Pe*-S2**	P_{max}, mL/g_{VS}	355.29 ± 2.97	347.93 ± 3.75	278.87 ± 2.52
	R_{max}, mL/g_{VS}/d	35.45 ± 0.72	34.62 ± 0.89	34.44 ± 0.88
	λ, d	0.35 ± 0.09	0.48 ± 0.11	0.32 ± 0.10
	R^2	0.9979	0.9965	0.9964
***Po*-S1**	P_{max}, mL/g_{VS}	294.36 ± 4.02	301.42 ± 6.67	300.65 ± 6.78
	R_{max}, mL/g_{VS}/d	35.70 ± 1.40	28.77 ± 1.45	28.11 ± 1.46
	λ, d	0.48 ± 0.15	0.67 ± 0.22	0.47 ± 0.24
	R^2	0.989	0.984	0.9825
***Po*-S2**	P_{max}, mL/g_{VS}	390.16 ± 4.63	307.15 ± 3.39	281.76 ± 2.02
	R_{max}, mL/g_{VS}/d	33.35 ± 0.76	38.43 ± 1.11	52.50 ± 1.29
	λ, d	0.27 ± 0.11	0.31 ± 0.11	0.21 ± 0.06
	R^2	0.9969	0.9947	0.9963
***Tv*-S1**	P_{max}, mL/g_{VS}	362.25 ± 4.77	251.23 ± 3.46	222.83 ± 2.85
	R_{max}, mL/g_{VS}/d	31.84 ± 0.96	31.37 ± 1.12	29.76 ± 1.06
	λ, d	0.00 ± 0.15	0.36 ± 0.13	0.23 ± 0.13
	R^2	0.9957	0.9925	0.9925
***Tv*-S2**	P_{max}, mL/g_{VS}	404.35 ± 6.46	250.64 ± 3.38	206.08 ± 2.65
	R_{max}, mL/g_{VS}/d	30.40 ± 0.71	33.96 ± 1.28	29.04 ± 1.10
	λ, d	0.00 ± 0.07	0.27 ± 0.13	0.15 ± 0.13
	R^2	0.9956	0.9916	0.9916
***Pe*-S1 (Cu^{+2})**	P_{max}, mL/g_{VS}	249.22 ± 6.65	296.46 ± 12.67	285.87 ± 8.75
	R_{max}, mL/g_{VS}/day	23.52 ± 1.29	19.07 ± 1.14	26.26 ± 1.56
	λ, d	0.78 ± 0.22	0.92 ± 0.29	1.03 ± 0.24
	R^2	0.9830	0.9820	0.9799
***Po*-S1 (+Cu^{+2})**	P_{max}, mL/g_{VS}	344.47 ± 10.30	299.34 ± 11.17	282.60 ± 7.48
	R_{max}, mL/g_{VS}/d	30.24 ± 1.74	28.08 ± 2.08	29.55 ± 1.76
	λ, d	0.76 ± 0.24	0.98 ± 0.29	0.76 ± 0.23
	R^2	0.9816	0.9689	0.9795

According to Table 3, values for the lag phase (λ) were lower than a day in all cases and presented slight variation for each strain. The assays with *Po* and *Pe*

presented a decrease of λ over time, with the values after 30 d of biological treatment being the lowest. On the other hand, the responses with the strain *Tv* were different. The results showed values of λ close to zero after 0 d of biological treatment; however, the lag phases after 30 d of biological treatment were lower than that after 15 d.

The maximum methane production rate (R_{max}) showed a different behaviour for each strain and particle size. For *Pe*-S1, the values increased with the time of biological treatment; however, for *Pe*-S2 this parameter remained constant. Regarding *Po*-S1, after 15 and 30 d of biological treatment, the values of R_{max} were similar and lower than those at 0 d. For *Po*-S2, R_{max} increased over time, and the values after 15 and 30 d were higher than those reported for S1. The values of R_{max} for *Tv*-S1 were similar; however, at S2 the higher value was reported at 15 d of biological treatment.

A previous research has indicated that maximum methane production rate on anaerobic digestion is related with substrate lignin content (Schroyen et al., 2015). Therefore, it is possible to explain the increase of R_{max} as function of fungal treatment time due to delignification for most of the studied cases. On the other hand, lignin degradation by different treatment types has associated inhibitory compounds released (Jönsson and Martín, 2016). Specifically, the application of an enzyme treatment on LW have shown the ability of release vanilic acid, 4-hydroxy-benzoic acid and p-coumaric acid but the concentration reached is lower than those reported as inhibitory on anaerobic digestion processes (Schroyen et al., 2015). In addition, the hydrolysates detoxification has

been reported for ethanol production (Jönsson et al., 1998) and for biogas production (Schroyen et al., 2017) due to enzyme treatment application. According to this background, it is possible to suppose a synergic effect provoked by fungal treatment on LW, due to the ability of lignin and phenolic compounds degradation by ligninolityc enzymes; that is to say, simultaneous delignification and detoxification.

For all the types of samples studied, biogas production reported by the S2 size was higher than that for S1. These results could be attributed to the different TL contents reported for both sizes, in which S1 had a higher content than S2 (section *3.1*). In addition, the weight and TL losses during biological treatment presented significant differences among particle sizes, where the TL loss was lowest with a small particle size (Table 2). A similar behaviour was reported previously, in which, using different particle sizes of sunflower oil cake, the degradation efficiency was higher for the large particle size due to a lower fibre content (De la Rubia et al., 2011). This common factor could explain why biogas production decreased for small particle sizes despite having more available specific surface for microorganisms. In addition, the decreased biogas could be attributed to the effect of particle size in the biological treatment stage because it affects the fungal penetration into biomass (Sindhu et al., 2016). The higher decrease in TL content and weight loss after 15 d and 30 d of fungal treatment using a small particle size could be attributed to the major fungal penetration capacity into corn stover through the outer cell wall of the waste; as a result, it could feed from the organic matter contained in LW such as cellulose and hemicellulose, reducing the organic matter amount available for the anaerobic

step. All these results are relevant because the behaviours obtained were related to the rapid growth observed for each strain, with *Tv* being the fastest followed by *Po* and *Pe*, in that order.

Furthermore, on the condition that the biogas production data is quantified using VS content values after 30 d of biological treatment, the experiments performed at small particle size showed a higher biogas production than large particle size (*Supplementary Material data of this work can be found at the end of the document*). This behavior agrees with the major capacity of fungal penetration at S1 and with the results obtained for TL loss and weight loss. However, the biogas production in absence of biological treatment and without thermal treatment; that is to say, the evaluation of size reduction treatment effect on raw material showed that the biogas production for S2 was the highest, being consistent with the differences of TL content reported for both particle sizes (*Supplementary Material data of this work can be found at the end of the document*). According to this background, it is possible to conclude that the biogas production using corn stover (LW) as substrate is favored at small particle size applying a biological treatment due to the fungal effect above the comminution effect.

4.3.2 Biogas production after biological treatment in the presence of inducer

In a previous section (*4.2.2.*), the increase in laccase enzyme production was shown using Cu^{+2} as an inducer. However, this dose of an external compound can affect the present stage of this process, biogas production. Figures 4.g and 4.h shows the biogas production curves obtained after biological treatment in the presence of the laccase inducer $CuSO_4$. The main result observed was the

decrease in biogas production by approximately 20 % compared with the same trial condition without the addition of copper for both strains. The response obtained for both fungi studied was predictable due to several reasons. First, copper belongs to the heavy metal group, and microorganisms that carry out acidogenesis and methanogenesis stages are sensitive to its concentration (Chen et al., 2008). Furthermore, several studies have demonstrated the inhibition by sulphate due to the production of sulphide in the AD process (Thanh et al., 2016).

The parameters obtained after data modelling provided more information for inducer effect interpretation (Table 2). In this case, the comparison of the maximum methane production rate (R_{max}) of the assays with and without laccase inducer showed a decrease by approximately 13.5 % due to the Cu^{+2} effect, showing the inhibition of the biogas production process. In addition, the values of the maximum biogas production (P_{max}) were lower in the presence of inducer by approximately 15 %.

Another parameter proposed to evaluate the process type is the selectivity index (Wan and Li, 2010). This ratio of lignin and cellulose removal during pre-treatment indicated the capacity of the selective degradation of lignin by applying white rot fungi. Previous studies have shown that the rice straw selectivity value obtained during fungal pre-treatment is directly proportional to the methane yield using *Po* and the soft rot fungi *Trichoderma reesei*. In addition, this study also found a weak relationship between the amount of lignin removed during pre-treatment and the increase in methane yield (Mustafa et al., 2016). Therefore, the methane yield

was not only dependent on lignin removal but also on the cellulose and hemicellulose amount remaining after pre-treatment. Considering the relationship between the selectivity index and methane yield presented in previous results, together with the results obtained in this study, it can be assumed that the selectivity index reached by the biological treatment was higher without the presence of the inducer due to the increase in enzyme production but decrease in methane yield. From this point of view, these results could provide interesting possibilities for the application of copper or other compounds application to change the fungi specificity.

Despite the results obtained, the overall effect of the inducer dosage should be quantified by an economic evaluation, in which the effect on enzyme production increase by 16 % and biogas production reduction by 20 %, as well as the cost of the copper dosage, were evaluated jointly.

5. CONCLUSIONS

Application of biological treatment with *Pe* is an alternative that improves biogas production and allows the production of laccase enzyme with high commercial value.

Copper as an enzyme inducer is an option to increase laccase production, but it reduces biogas production. The global behaviour of the system depends significantly on the particle size and structural characteristics of the waste.

Global results indicated the feasibility of carrying out a sequential strategy to produce extracellular enzymes and biogas using corn stover as raw material. However, further research must consider the extraction and purification stages for economical evaluation of the enzyme produced.

6. PRODUCTIVITY

- Paper: V. Wyman, J. Henríquez, A. Carvajal, C. Palma, "*Lignocellulosic waste valorisation strategy through enzyme and biogas production*", Bioresource Technology (2017), doi: https://doi.org/10.1016/j.biortech.2017.09.055

- Oral- Poster Presentation: "*Lignocellulosic waste valorisation strategy for enzyme and biogas production*" V. Wyman, C. Palma, A. Carvajal. IBBK– The International Conference Progress in Biogas IV, Universität Hohenheim, Stuttgart, March 2017

- Poster Presentation: "*Application of a biological pretreatment of corn stover by two white rot fungi*" V. Wyman, D. González, C. Palma, A. Carvajal. DAAL– The XII Latin American Workshop and Symposium on Anaerobic Digestion, Cusco, October 2016

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Supplementary Material

S1. Effect of thermal treatment in corn stover (sterilization stage)

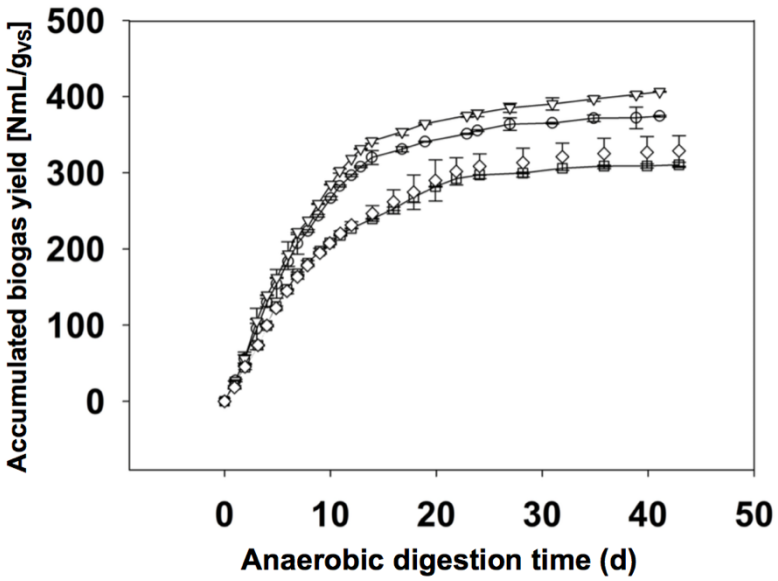
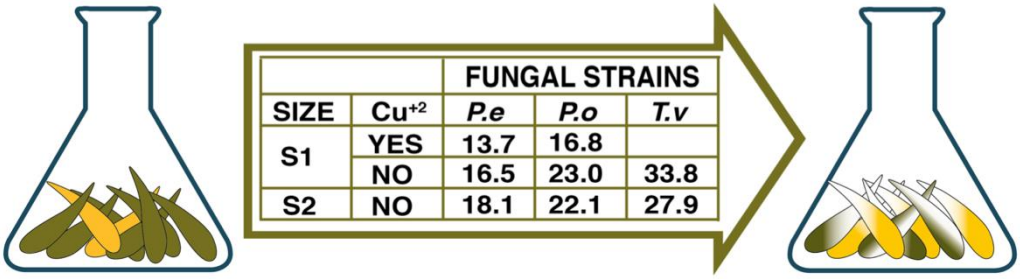


Figure S1: Accumulated biogas yield of corn stover before and after thermal treatment (TT). Symbols: triangle, S2 before TT; circle, S2 after TT; diamond, S1 before TT; square, S1 after TT.

As an additional analysis, it was considered necessary to add information about the sterilization effect on the waste. Technically, the sterilization process is a thermal pre-treatment, carried out at 120 ºC for 30 min; thus, the waste matrix could be affected. According to Figure S1, it is possible that biogas production was not favoured by the only action of thermal pre-treatment carried out before the biological treatment. These results obtained were similar for both sizes, S1 and S2. However, the waste at the higher size (S2) presented greater biogas production in raw conditions, without the effect of sterilization and biological treatment. A similar behaviour was observed with thermal pre-treatment on rice straw and maize stalks at 90 °C and 120 °C, respectively, where no significant improvement in the methane yields was reported compared with untreated

samples (Menardo et al., 2012). Commonly, thermal pre-treatment is indicated as effective for AD improvement and increased methane production (Ward et al., 2008); however, this type of pre-treatment above 160 °C also produces inhibition or toxicity in the participant microorganisms of the process due to the release of different compounds such as phenols (Hendriks and Zeeman, 2009). In particular, considering that the thermal pre-treatment did not improve the anaerobic digestion process for the waste under study. The global results obtained for enzyme and subsequent biogas production showed that the proposed process could be a better alternative for AW treatment, allowing the increase in the waste value

S2. Volatile solid content reduction after 30 d of biological treatment



		FUNGAL STRAINS		
SIZE	Cu^{+2}	*P.e*	*P.o*	*T.v*
S1	YES	13.7	16.8	
	NO	16.5	23.0	33.8
S2	NO	18.1	22.1	27.9

Figure S2: Volatile solid content reduction after 30 d of biological treatment (%).

S3. Biogas yield obtained with VS content after 30 d of fungal treatment

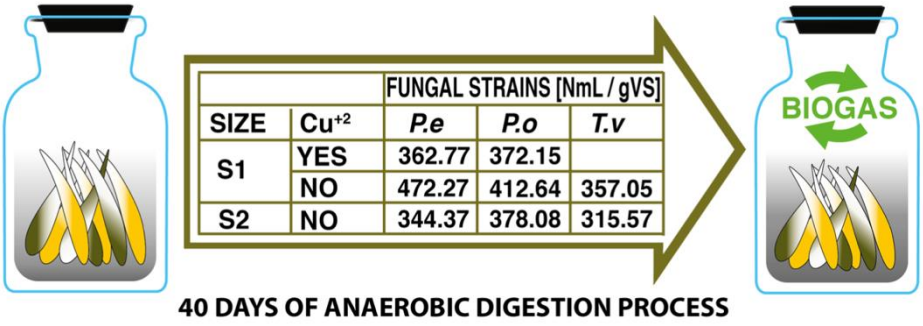


Figure S3: Biogas yield obtained with VS content after 30 d of fungal treatment.

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