Title

Inhibitory effects of *p*-coumaric acid on the biogas production process, and the interaction with an enzymatic pretreatment.

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Highlights

- Hydrolysation speed of anaerobic digestion of miscanthus is decreased by *p*-coumaric acid.
- Versatile peroxidase is inhibited by *p*-coumaric acid.
- Laccase treatment reduces the concentration of *p*-coumaric acid.

Keywords

p-coumaric acid, inhibition, laccase, versatile peroxidase, anaerobic digestion, lignocellulose

Abstract

The impact of *p*-coumaric acid on anaerobic digestion of lignocellulosic biomass (hemp straw and miscanthus) was studied. Biogas production during anaerobic digestion is known to be inhibited by phenolic compounds. Addition of *p*-coumaric acid in various concentrations: 0, 100, 500 and 2000 mg/l showed a difference in inhibition between the substrates. Hydrolysation speed in anaerobic digestion of miscanthus was inhibited up to 33 % by 2000 mg/l of *p*-coumaric acid. The initial rate of biogas production during the anaerobic digestion of hemp straw was not inhibited. Miscanthus consists of more lignin, making it less accessible, in combination with a higher concentration of phenolic compounds released after harsh pretreatments, inhibition levels during the anaerobic digestion can be reached.

An enzymatic pretreatment can polymerize the inhibiting phenolic compounds, as well as degrade the lignocellulose structure. The interaction of laccase and versatile peroxidase individually and *p*-coumaric acid was therefore studied. Hemp straw and miscanthus was incubated with 0, 100 and 500 mg/l of the phenolic compound with laccase or versatile peroxidase for 0, 6 and 24 hours. Laccase successfully removed the phenolic component, versatile peroxidase was inhibited by 100 mg/l of *p*-coumaric acid. A decrease of phenolic compounds by laccase pretreatment can lower the inhibition of biogas production considerably.

1. Introduction

Anaerobic digestion is a four step production process, the rate-limiting hydrolysis, acidogenesis, acetogenesis and methanogenesis [1]. The hydrolysation speed can be increased by removing or degrading lignin [2]. Schroyen et al. demonstrated that the initial rate of biogas production during the first 7 days of the anaerobic digestion is related to lignin concentrations [3].

Numerous pretreatment techniques, including physical, chemical, physicochemical, and biological pretreatment methods degrade lignocellulose. A pretreatment facilitates hydrolysation, avoids formation of fermentation inhibitors, i.e. organic acids, furan

derivatives and polyphenolic compounds, and sugar degradation products [4]. Oxidative methods fragment and oxidizes lignin to carboxylic acids and phenolic compounds [5]. Ionic liquid pretreatment does not result in high inhibitory levels, allthough remaining ionic liquids are possible toxic to enzymes and microorganisms [6]. During acid-based chemical pretreatment, acids from hemicellulose are set free. Degradation of uronic and acetic acid may lead to 5-hydroxymethyl-2-furaldehyde and furfural production, other acids like formic and levulinic acids are formed from sugar degradation [7]. Other studies of acidic pretreatments determined the formation of phenolic compounds (i.e. 4-hydroxybenzoic acid, vanillin, syringic acid) [8]. Some physical pretreatments, like milling, do not release these inhibiting compounds [9]. However the energy demand is too costly to get an economically feasible process [10].

Biological pretreatments have the advantage of producing fewer inhibiting compounds and are a low cost alternative to more severe, energy consuming pretreatment methods. Schroyen et al. described several individual phenolic compounds which were obtained after enzymatic pretreatment with laccase and versatile peroxidase on a set of various substrates [3]. *P*-coumaric acid was among the most prominent ones released. However the concentrations reached after enzymatic treatment were not close to the inhibiting levels of anaerobic digestion (>1000 mg/l) [11-12]. Other types of pretreatments such as, thermal, acidic or alkaline pretreatments, release higher values of these phenolic compounds which could inhibit the preceding biodegradation steps.

A detoxifying biological pretreatment can be introduced to counter these inhibiting compounds [13]. This detoxifying effect of laccase has been noted by Jönson et al., however the impact of individual phenolic compounds on the lignin degrading enzymes is unknown and is an important factor in creating an efficient enzymatic pretreatment step [14].

In this study the interaction between lignin degrading enzymes, more specific versatile peroxidase and laccase, and *p*-coumaric acid was tested at several concentration levels during incubations of 0, 6 and 24 hours. Besides that the impact of different concentrations of *p*-coumaric acid on the anaerobic digestion of hemp straw and miscanthus was studied.

2. Materials and methods

2.1 Chemicals

Sulfuric acid (H_2SO_4), ethanol and Folin-Ciocalteu's phenol reagent were attained from ChemLab (Zedelgem, Belgium). *o*-Coumaric acid, *p*-coumaric acid, sodium chloride (NaCl), sodium malonate ($C_3h_2O_4Na_2$), hydrogen peroxide (H_2O_2), 2,2 azinobis (3-ethylbenzthiazoline)-6 sulphonate, sodium citrate, veratryl alcohol and laccase enzyme were acquired from Sigma-Aldrich (Bornem, Belgium). Sodium carbonate (Na_2CO_3) was purchased from Merck (Darmstadt, Germany), and 1-hydroxybenzotriazole was obtained from Janssen Pharmaceuticals (Beerse, Belgium). HPLC-grade methanol (MeOH), HPLC-grade water, acetic acid, citric acid and potassium sodium tartrate ($KNaC_4H_4O_6$-$4H_2O$) were acquired from VWR (Leuven, Belgium), while versatile peroxidase was obtained from Jena Bioscience (Jena, Germany). All chemicals were used as provided.

2.2 Substrates

Miscanthus (*Miscanthus giganteus*) was acquired from the Institute of Agricultural and Fisheries Research (Merelbeke, Belgium), while hemp straw (*Cannabis sativa L*.) was obtained from InAgro vzw (Roeselare, Belgium).

2.3 Experimental Setups

2.3.1 Effect of lignin degrading enzymes on phenolic compounds

Two grams of substrate, miscanthus or hemp straw, were incubated in 40 ml acetate buffer (0.1 M, pH = 4.5) with different concentrations (0, 100 and 500 mg/l) of *p*-coumaric acid (p-CA) for 24 hours. Versatile peroxidase (VP, 1.5 U/g biomass; 1 U is defined as the release of 1 µmol Mn(II)/min at pH 4.5 and 25°C) was added to one series, laccase (LA, 2 U/g biomass; 1 U is defined as the release of 1 µmol Catechol/min at pH 6 and 25°C) was added to a second series. Samples of the liquid were taken after 10 minutes, 6 hours and 24 hours of incubation. Blank incubation, i.e. buffer with the various concentrated phenolic compounds, pretreated with LA or VP were included. The concentration of the phenolic compounds of all

samples and the laccase activity in the laccase treated samples was determined. Three independent replicates were performed.

2.3.2 Impact of phenolic compounds on anaerobic digestion

Two grams of hemp straw and miscanthus were added to 40 gram of sludge so to keep a substrate to inoculum ratio of 0.5 g VS/ g VS. p-CA was added at concentrations of 0, 100, 500, 1000 and 2000 mg/l to the anaerobic digestion inoculum. The reactors (250 ml) were shaken and connected to a water-displacement system. The volume of displacement was used to calculate the biogas production in norm liter under standard temperature and pressure [15]. Next to this, the inoculum with the different concentrations of phenolic compounds without substrate, were started up simultaneously as control and daily biogas production was measured over 30 days [16]. For each treatment three independent replicates were done.

2.3.3 Impact of LA-treated p-CA on anaerobic digestion

p-CA was added to an acetate buffer (0.1 M, pH=4.5) in concentrations of 0 and 500 mg/l. Two grams of substrate, miscanthus or hemp straw, was added. One series of samples was added to the aforementioned inoculum, and the biogas production was determined for over 30 days. Another series was first pretreated with laccase for 24 hours at 30°C. Buffer with a concentration of 0 and 500 mg/l of p-CA were also anaerobically digested for 30 days without addition of any substrate as a base. All incubations were performed independent in triplicate.

2.4 Analysis

2.4.1 Phenolic Compounds

To determine the total phenolic compounds in the liquid, the Folin-Ciocalteau method was used as described by Singleton et al. and executed as described in Schroyen et al. [16-17]. A specific standard curve was used based on p-CA, since p-CA showed different absorbance

159 values than gallic acid. The concentration of total phenolic content in the liquid were
160 measured at the start, after 6 and 24 hours. One of the three repetitions was measured with
161 the HPLC as a confirmation of the folin experiment. The HPLC experiment was performed as
162 described in Schroyen et al. [16]. To quantify the results the measured concentration was
163 calculated relatively to the initially added concentration of phenolic compound at the start.
164 RC = 100 * MC / IAC (RC = relative concentration (%); MC = measured concentration (mg/l);
165 IAC = initially added concentration (mg/l)).
166
167 *2.4.2 Enzymatic Assays*
168
169 Versatile peroxidase activity was measured in a mixture of 1 mL 30mM veratryl alcohol, 1
170 mL citrate buffer (0.1 M, pH = 3), with or without a phenolic compound at a concentration
171 of 100 g/ml and 0.5 mL 20mM H_2O_2. The difference in absorbance was measured every 30
172 seconds over 150 seconds at 310 nm (ε_{310} = 4600 M cm^{-1}) [18].
173 In an assay 2,2 azinobis (3-ethylbenzthiazoline)-6 sulphonate (ABTS) was oxidized by laccase
174 at 35 °C. The laccase activity assay was performed in a 1.5 mL mixture of 0.5 ml of 1 mM
175 ABTS, 0.5 ml of acetate buffer (0.1 M, pH = 4.5) and 0.5 mL enzyme solution or sample. The
176 laccase activity was measured spectrophotometrically at 420 nm (ε_{420} = 36000 M cm^{-1}) after
177 5 minutes [19]. The enzymatic activity in the samples was reported relatively to the laccase
178 activity determined in the solution with only buffer, laccase and ABTS.
179
180 *2.5 Statistical Analysis*
181
182 For all statistical analysis IBM® SPSS® Software version 23 was used. A two-way ANOVA was
183 executed on all data. The effect of pretreatment duration, type of phenolic component and
184 the starting concentration of the phenolic component on the relative concentration of
185 phenolic components after a treatment with laccase or versatile peroxidase was studied. In
186 a second analysis the effect of the initial concentration of phenolic compounds, the
187 pretreatment duration and the substrate on the enzyme activity was determined. The *p*-
188 values are given throughout the article with a significance level of 0.05.
189 A first-order model $P = \gamma * (1-e^{(-\mu t)})$ [16,20] was created and showed the biogas production
190 as a function of time (t(d)). An exponential rise, characterized by the specific production rate

μ (d^{-1}) was assumed to a maximum biogas yield given by γ (Nl/kg VS). The model was fitted using Microsoft Excels solver to minimize the sum of squares of differences between the model and the measured biogas production. The initial slope was based on the first 7 days of the fitted model and used as a measure of hydrolysation rate.

3. Results and discussion

3.1 Impact of enzymatic pretreatment on phenolic compounds

In a first step, the impact of an enzymatic pretreatment (laccase or peroxidases) on p-CA in the presence of different lignocellulose substrates was investigated. In figure 1 laccase treated samples are presented, the concentration of the phenolic compound is given relatively to the initially added concentration for the different pretreatment durations and used substrates. A detoxifying effect is noticed, as p-CA was found in relatively low amounts (20 % of the original amount added) after an incubation of 24 hours with laccase. Laccase are blue copper oxidases, which catalyze one-electron oxidation of phenolic compounds, and thus in absence of the other enzymes it should have the ability to polymerize the phenolic units. The detoxifying effect of laccase was also seen by Jönsson et al. [14]. Versatile peroxidase contrary to laccase did not result in any change in concentration of phenolic compounds over time (Figure 2).

3.2 Impact of phenolic compounds on lignin degrading enzymes

The effect of phenolic compounds on laccase and versatile peroxidase is less studied compared to the enzymatic influence on inhibiting compounds [21-22]. Bollag and Leonowicz studied the inhibition of laccase but did not report on phenolic compounds [23]. The activity on hemp straw and miscanthus (Figure 3) was measured relative to the activity in buffer with neither substrate, nor phenolic compound. A link between the decrease in concentration of a phenolic compound due to laccase and activity of laccase was seen. A gradual decrease of phenolic inhibitors is reached after 24 hours of incubation (Figure 1), meaning the difference in enzyme activity between the different concentrations has disappeared, while the activity of laccase at the start and after 6 hours of incubation is

decreasingly inhibited (Figure 3), indicating a clear association between results of both experiments. A trend ($p < 0.1$) was found in the samples of 0 and 100 mg/l of phenolic compounds compared to the samples with a concentration of 500 mg/l of phenolic compounds. These results indicate a clear association between results of both experiments, removal of phenolic content after 24 hours results in a significant ($p < 0.05$) higher laccase activity.

The activity of versatile peroxidase could not be measured. Due to the non-transparency samples, there was simply too much interference. However versatile peroxidase activity was measured in the buffer and after addition of 100 mg/l of p-CA to the buffer. An increased absorbance was measured over time, and the slopes of this increase were calculated. The slope in the buffer was 0.136 U/min, versatile peroxidase was inhibited entirely by p-CA, this was shown by the resulting slope of -0.011 U/min. This agrees with the results from figure 2, where no decrease of p-CA was detected after a treatment with versatile peroxidase.

3.3 Impact of phenolic compounds on the biogas production

Kayembe et al. tested the anaerobic digestion of acetic acid neutralized to pH 7, enriched with macro-nutrients, i.e. NH_4Cl (170 g/l), KH_2PO_4 (37 g/l) [12]. Addition of 1249 mg/l phenol resulted in a 50 % inhibition of methanogenic activity. This inhibition is caused by the loss of integrity of biological membranes and thus reducing cell growth and sugar assimilation [24]. Hernandez and Edyvean have tested seven phenolic compounds inhibiting the anaerobic biogas production from the digestion of D-glucose, yeast extract and nutrient broth [11]. To reach an inhibition of 50 %, an addition of 120 mg phenol, 328 mg catechol or 271 mg 4-hydroxybenzoic acid per gram VSS biomass was required. The toxicity of phenolic compounds is, even in smaller concentrations, more severe than furfural and hydroxymethylfurfural [25]. These studies show that an accumulation of inhibiting phenolic compounds after pretreatment need to be removed or immobilized. Therefore, the aim of this experiment was to study the impact of individual phenolic compounds on lignocellulose substrates.

The impact on the hydrolysation speed was determined by calculating the slope of the biogas curve in the first 7 days. The slopes of the anaerobic digestion of the samples with miscanthus are given in figure 4, and of the samples with hemp straw are given in figure 5. Miscanthus has a higher lignin concentration than hemp straw [3], it is less accessible for degradation and has a lower slope in biogas production compared to hemp. These slopes are shown in figure 4 where an inhibition is noted initially at 100 mg/l of the added phenolic component, and increasing the concentration of the phenolic compounds resulted in an increased inhibition level. Hemp straw is inhibited less, a small decrease of rate in biogas production was only seen in samples where 2000 mg/l of p-CA was added.

3.4 Detoxification effect on biogas production

Figure 1 shows a detoxification effect of laccase, removing up to 80 % of the concentration of added p-CA. In Figure 4 inhibition of anaerobic digestion of miscanthus was observed due to p-CA. At 100 mg/l a drop in the hydrolysation rate (Nl/kg VS/d) of 20 % was noticed, while at 1000 mg/l the biogas production dropped to 60 %. Laccase treatment of 24 hours reduced greatly the added p-CA (Fig. 1). To estimate the detoxifying potential of laccase, biogas production of samples supplemented with 500 mg/l p-CA with and without a 24 hours laccase treatment were measured (Figure 6). In general, the more lignin containing miscanthus had a lower rate of biogas production. The untreated samples declined with addition of p-CA, while laccase pretreated miscanthus showed no inhibition of the initial biogas production by the phenolic component. Pretreatment of hemp straw show a small increase compared to the untreated samples.

Looking at the final biogas production a decrease of total biogas produced was seen in the untreated samples, if the phenolic component is added (Fig. 7). Pretreated samples with 500 mg/l p-CA did not show this decrease.

4. Conclusion

To remove the toxic effect of the phenolic compounds an incubation with laccase can be proposed. This incubation can be an important detoxifying step in a multi-step pretreatment process, removing produced inhibitors after a faster and harsher pretreatment. Phenolic

compounds are inhibiting the activity of versatile peroxidase and also have an impact on the initial hydrolysation rate during anaerobic digestion. The effect is clearly seen for lignin rich substrates. The microbial community can overcome the inhibition after 30 days and reach a similar bio methane potential over time.

Acknowledgements

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Figure 1: The relative concentration of phenolic compounds found in the liquid fraction of the initial added concentration of 100 and 500 mg/l of *p*-coumaric acid after various incubation durations with laccase. (n=3)

Figure 2: The relative concentration of phenolic compounds found in the liquid fraction of the initial added concentration of 100 and 500 mg/l of *p*-coumaric acid after various incubation durations with versatile peroxidase. (n=3)

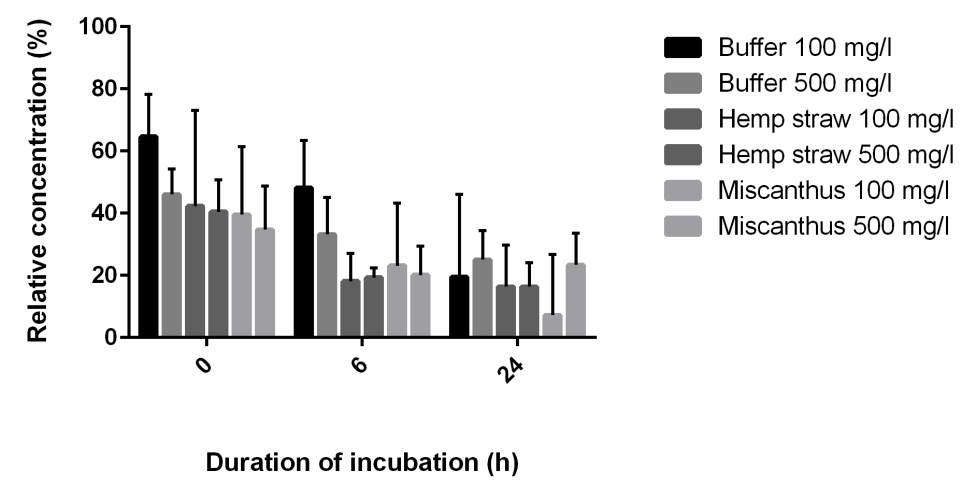
Figure 3: The laccase activity of the various samples incubated with 0, 100 and 500 mg/l of *p*-coumaric acid, relative to laccase activity measured in a buffer without inhibiting compounds. (n=3)

Figure 4: The initial slope of biogas production of miscanthus as substrate with various added concentrations of *p*-coumaric acid. (n=3).

Figure 5: The initial slope of biogas production of hemp straw as substrate with various added concentrations of *p*-coumaric acid. (n=3).

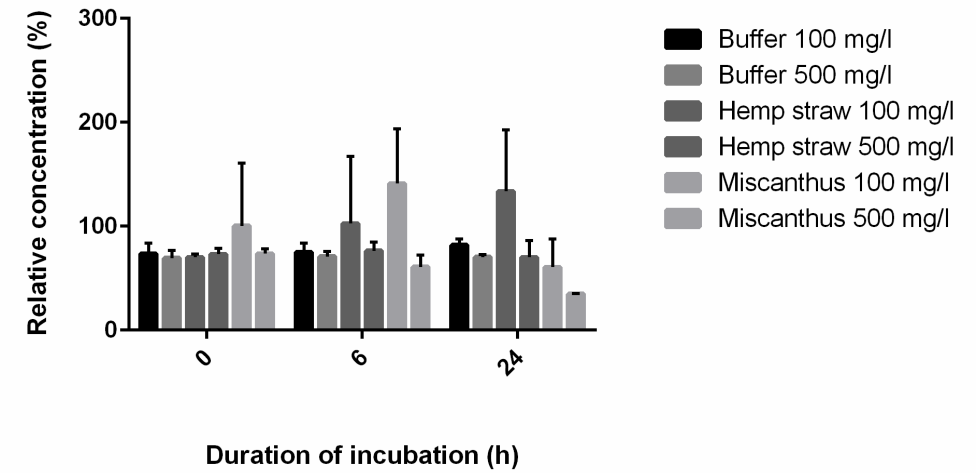
Figure 6: The initial slopes of the biogas curves after 7 days of the substrates with added *p*-coumaric acid concentrations (0, 500 mg/l) with and without a 24 hour treatment with the laccase enzyme. (n=3).

Figure 7: The bio methane potential (BMP) of the substrates with added *p*-coumaric acid in concentrations 0 and 500 mg/l with and without a 24 hour treatment with the laccase enzyme. (n=3).



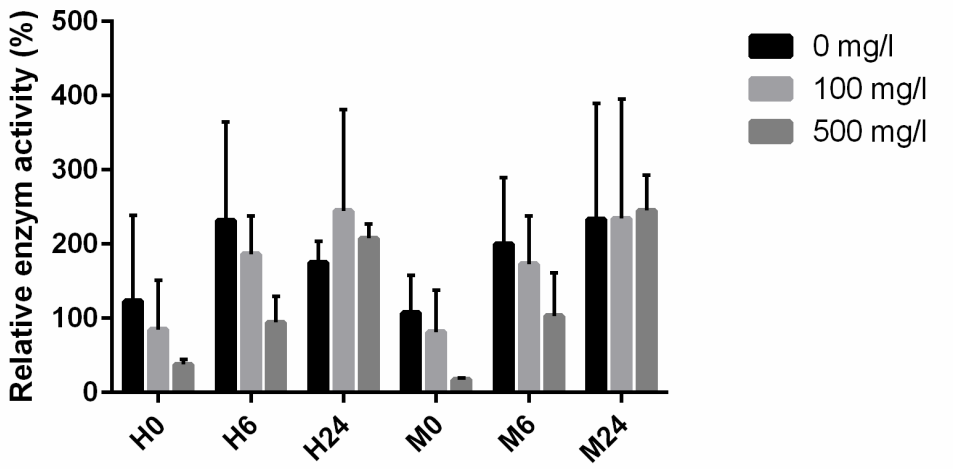
406

407 **Figure 1: The relative concentration of phenolic compounds found in the liquid fraction of the initial**
408 **added concentration of 100 and 500 mg/l of *p*-coumaric acid after various incubation durations with**
409 **laccase. (n=3)**



410

411 **Figure 2: The relative concentration of phenolic compounds found in the liquid fraction of the initial**
412 **added concentration of 100 and 500 mg/l of *p*-coumaric acid after various incubation durations with**
413 **versatile peroxidase. (n=3)**



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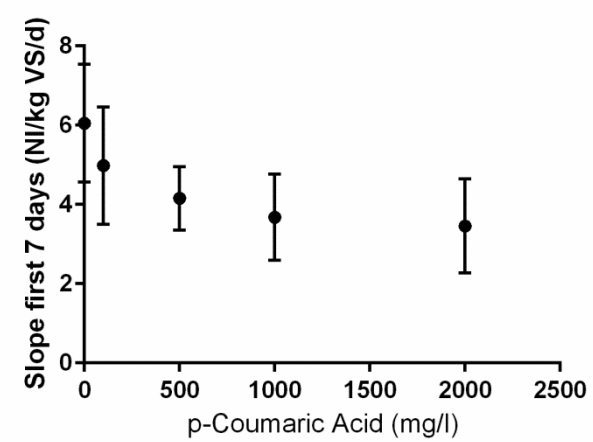
415 **Figure 3: The laccase activity of the various samples incubated with 0, 100 and 500 mg/l of *p*-coumaric**
416 **acid, relative to laccase activity measured in a buffer without inhibiting compounds. (n=3)**



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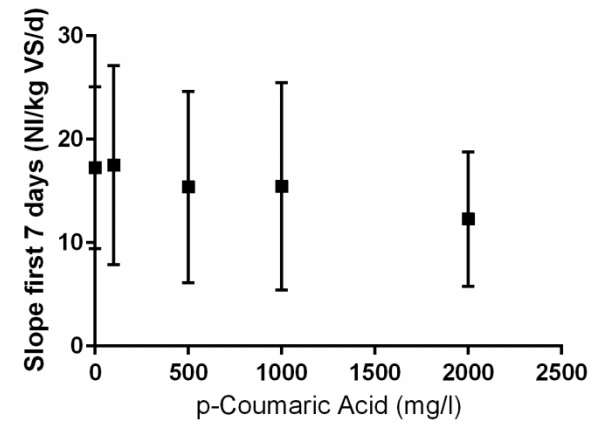


Figure 5: The initial slope of biogas production of hemp straw as substrate with various added concentrations of *p*-coumaric acid. (n=3).

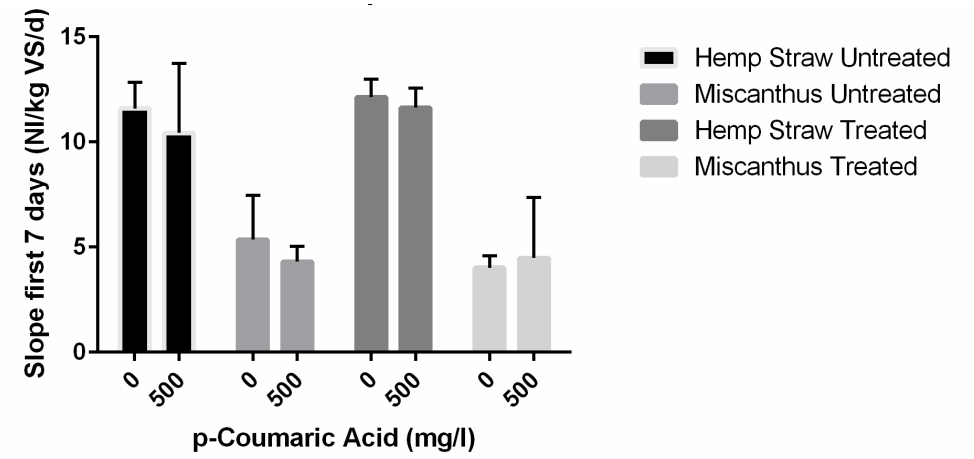
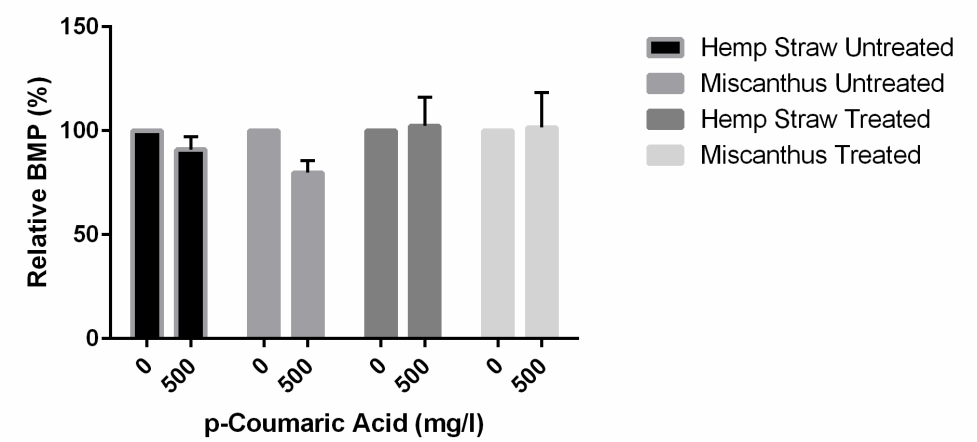


Figure 6: The initial slopes of the biogas curves after 7 days of the substrates with added *p*-coumaric acid concentrations (0, 500 mg/l) with and without a 24 hour treatment with the laccase enzyme. (n=3).



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