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Modeling and optimization of polyethylene glycol (PEG) addition for cost-efficient enzymatic hydrolysis of lignocellulose

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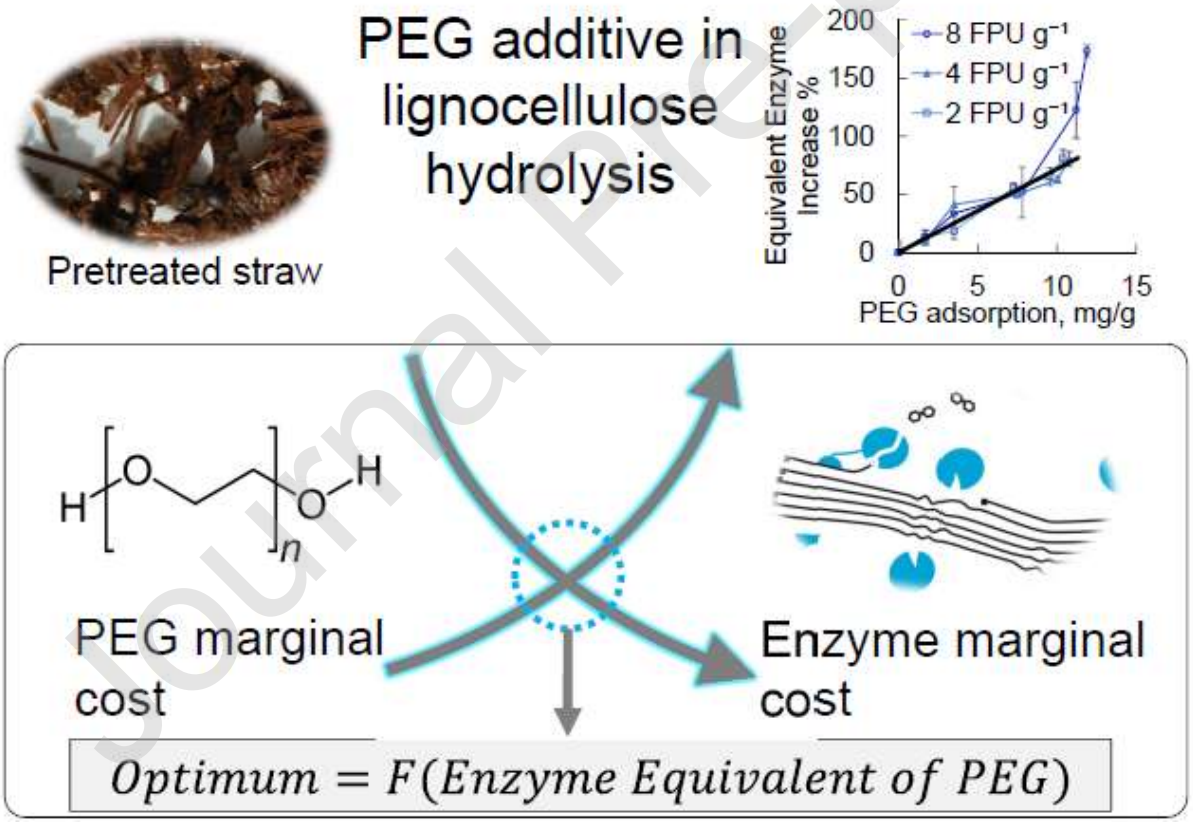
Graphical abstract



HIGHLIGHTS

- Model for enzyme equivalent of PEG addition was formulated
- %-increase in apparent enzyme dosage by PEG did not depend on absolute enz. dosage
- %-increase in apparent enzyme dosage correlated linearly with PEG adsorption
- Cost-optimization of PEG addition was carried out by marginal analysis

ABSTRACT

Enzyme consumption is a key cost in the lignocellulosic sugar route for production of biofuels and chemicals, and polyethylene glycol (PEG) is a low-cost additive that improves hydrolysis efficiency. Despite many studies in this area, the relation of benefit over cost of PEG addition remains unclear. This article describes principles for expressing the effect of PEG as an equivalent enzyme amount, by merging PEG adsorption kinetics with a standardized enzyme dosage response. This model allows cost optimization of PEG addition by marginal analysis, as a function of enzyme dosage, solids concentration and price-ratio of enzyme and PEG. The model is based on the novel observations that the relative increase in apparent enzyme dosage by PEG addition is constant regardless of the absolute enzyme dosage, and that the increase correlates linearly with adsorption of PEG on hydrothermally pretreated wheat straw. The optimum ranged for most cases between 7 and 14 mg PEG per g substrate. The addition of PEG was attractive only above a threshold price-ratio, which decreased from 7.0 to 1.4 as enzyme dosage increased from 2 to 10 cost units per g substrate, showing that the incentive for introducing PEG to the process becomes stronger as the enzyme dosage increases.

Keywords: Wheat straw; Cellulase; Hydrolysis additive; Langmuir; Nonproductive binding; Marginal benefit

1. INTRODUCTION

In the recent decades, enzymatic hydrolysis of lignocellulose materials into sugars has been vigorously studied for the production of renewable biofuels and chemicals by fermentation. Although different pretreatments have been developed for allowing efficient enzymatic hydrolysis of cellulose, enzyme consumption still remains a major challenge for the process economy [1]. Additives, such as non-ionic surfactants and proteins have been found to improve hydrolysis [2,3], and one of the most frequently studied surfactants is polyethylene glycol (PEG), due to its effectiveness and low price.

The effect of surfactants has been particularly related to lignin-containing materials, such as those produced by hydrothermal or dilute acid pretreatments and steam explosion, while the effect has been small for delignified lignocellulose substrates [2–10]. Simultaneously with the improved hydrolysis, decreased adsorption of cellulases on the material has been observed. Furthermore, Sewalt *et al.* [9] showed that addition of lignin to a suspension of cellulosic fibres had a major adverse effect on hydrolysis, which was reversed by the addition of PEG. Strong evidence therefore indicates that the major mechanism of the effect of PEG on hydrolysis is adsorption of PEG on lignin, thus reducing non-productive binding and inactivation of enzymes [8,11]. The adsorption of PEG on lignin is understood to result from a combination of hydrophobic interactions and hydrogen bonding with unsubstituted phenolic hydroxyls [3,5,7]. PEG with molecular weight above 2000 g/mol has been shown to be effective for improving hydrolysis and the effect depends on the dosage per substrate dry matter (DM) rather than its aqueous concentration [4,12]. Increasing temperature increases the adsorption of PEG on lignin-containing materials, as well as its positive effect on hydrolysis [5,11]. This has been interpreted to result from increased hydrophobic interactions between PEG and lignin at higher temperatures, and is in accordance with the similarly temperature-dependent adsorption and inactivation of cellulases on lignin [13,14]. In addition to blocking of lignin, additional effects have been suggested for explaining a positive effect of PEG with some lignin-free celluloses [15,16], including increased water activity [16], thermal stabilization of enzymes [17], and a direct effect of PEG on the substrate hydrolyzability [4,12].

In order to assess the potential of an additive to improve the economic viability of lignocellulose hydrolysis, it is crucial to weigh the cost of the additive against its effect on the cost of enzymes. However, this relationship is obscured by the nonlinear correlation of hydrolysis yields with

enzyme dosage [18]. Although the mechanisms behind the effect of PEG have been extensively studied, the reports of the economic potential of PEG have so far only been based on single estimates of enzyme savings [19–22], whereas general means for converting PEG into equivalent enzyme savings at variable hydrolysis conditions have not been presented. Vaidya *et al.* [19] presented kinetic projections of PEG adsorption together with empirical hydrolysis modelling, but no link was presented between the models. In the absence of a continuous relation between PEG addition and enzyme consumption, evaluation of the marginal benefit against the marginal cost of PEG addition has not been possible. If adsorption of both PEG and cellulases on lignin were irreversible, the amount of enzymes salvaged by PEG could be simply proportional to the lignin surface area covered by PEG, and a constant conversion factor would exist for the enzyme equivalent of PEG. However, the adsorption of PEG is expected to be reversible, since it has been shown to follow Langmuirian adsorption kinetics [5,12,19]. Furthermore, interactions between cellulases and lignin comprise a complicated combination of reversible [23] or irreversible [24] binding and inactivation on lignin surface, potentially by unfolding of the protein structure [14,25], for which the kinetics are unclear. Therefore the enzyme equivalent of PEG is expected to follow a more complex dependence on PEG and enzyme loading, which has so far not been resolved. Furthermore, the changes in PEG adsorption kinetics in the course of hydrolysis are not known.

This article presents a model for enzyme savings achieved by PEG addition in hydrolysis of hydrothermally pretreated wheat straw, and application of the model for the first reported cost-optimization of PEG dosage by marginal analysis. By using a non-linear hydrolysis standard, the apparent increase in enzyme dosage by PEG addition was determined and combined with the adsorption kinetics of PEG. This allowed constructing a model describing the enzyme equivalent of PEG. The model was then applied for simulating enzyme savings by PEG addition and the dependence of the optimal PEG loading on the price-ratio of enzymes and PEG. Furthermore, the change in PEG adsorption kinetics in the course of hydrolysis was determined and direct effects of PEG on substrate hydrolyzability and enzyme stability were further evaluated.

2. MATERIALS AND METHODS

2.1 Materials

Wheat straw from Finland was hydrothermally treated (HT) at 180–190 °C, for 20 min, steam exploded and washed as described previously [26]. According to compositional analysis [27], the HT-straw was composed of 54.1% glucan, 4.1% xylan 0.3% arabinan, 27.4% lignin and 2.2% ash. Whatman 1 filter paper (Sigma-Aldrich) was ground using a Fritsch Pulverisette rotor mill (Fritsch, Germany) to pass a 1 mm screen, in order to enable homogenous sampling. Polyethylene glycol 4000 with M_r 3500-4500 g/mol (Fluka 81240) was used as an additive in hydrolysis experiments.

GreenValue lignin (Protobind 1000) was purchased from GreenValue SA (Switzerland) and has been previously found to contain 91.6% lignin, 3.9% carbohydrates, and 1.4% ash. The hydroxyl groups consisted of (mmol g^{-1}) aliphatic (1.92), carboxylic (1.18), and phenolic (2.55) functionalities [28]. GreenValue lignin (2.0 g) was acetylated in 40 mL of pyridine:acetic anhydride (1:1, v:v) mixture at room temperature during 40 h. Acetylated lignin was purified by dispersing solid material in a series of solvents which were removed by evaporation under reduced pressure. The order of the solvents used was methanol (25 mL), toluene (three times 10 mL), and methanol (25 mL). The acetylation reaction was confirmed to be successful by ^{31}P NMR spectroscopy, according to Granata and Argyropoulos [29].

2.2 PEG adsorption studies

Adsorption studies of PEG were carried out for hydrothermally treated wheat straw, Whatman 1 paper, GreenValue lignin and acetylated lignin at 2% or at 0.4% solids concentration. The studied PEG dosages varied depending on the adsorption capacity of the materials and were between 0 and 10% of DM for HT-straw and filter paper and 0 and 100% for GreenValue lignin and acetylated lignin. Duplicate reactions were performed in 15 ml polypropylene tubes in 0.1 M Na-phosphate buffer, pH 5.0. The suspension was preheated at 50 °C for 1 hour before addition of PEG. After PEG addition, tubes were shaken at 200 rpm for 2 hours. The suspensions were centrifuged to separate the solids. The concentration of unadsorbed PEG was analyzed from the supernatants by high-performance size-exclusion chromatography (HPSEC), as described below.

2.3 Hydrolysis experiments

All hydrolysis reactions were performed in duplicate at 50 °C, pH 5 (0.1 M Na-phosphate buffer), 200 rpm at a solids concentration of 5%. A mixture of enzymes [26] consisting by volume of 85% cellulase (Econase CE, Roal Oy), 10% β-glucosidase (Novozyme 188) and 5% xylanase (GC 140, Genencor) was used. The cellulase activity of the mixture was 51.0 FPU g^{-1} (filter paper units) and the protein concentration was 42.9 mg ml^{-1} according to Bradford assay against bovine serum albumin standards (Bradford reagent, BioRad). Tetracycline (4 mg ml^{-1}) and cycloheximide (3 mg ml^{-1}) were added to prevent microbial contamination.

The effect of PEG on hydrolysis yield was determined by hydrolysis of hydrothermally pretreated wheat straw at 5% solids concentration (w/w), in a total volume of 5 ml in 15 ml polypropylene tubes. PEG was added to the mixture (0, 2, 5, 10, 20 and 50 mg g^{-1} substrate DM) and preincubated for 2 h at 50 °C, shaken at 200 rpm in a tilted position before enzyme addition (dosages of 2, 4 and 8 FPU g^{-1} DM) and hydrolysis for 18 h. After the reaction, the suspension was centrifuged and the supernatant was analyzed for sugars and PEG. The repetition at 8 FPU g^{-1} with 5 mg g^{-1} PEG was discarded as an outlier due to strong deviation from general trend. PEG concentration was analyzed from single hydrolysates, which was sufficient for determining the standard error of the adsorption isotherm parameters.

A non-linear hydrolysis standard was constructed by fitting a rational function, describing asymptotic increase towards the value of a (Eq. 1), to hydrolysis yield Y (%) as a function of enzyme dosage E (FPU g^{-1}) by nonlinear regression (function *nlinfit*, Matlab 2015a, Mathworks), with fitting parameters a = 90.0 (%) and b = 0.128 (g FPU^{-1}) (R^2 = 0.996). Solving E from the equation as a function of Y allowed calculation of apparent enzyme dosage E_{app} from yield Y obtained with PEG addition. The %-increase in apparent enzyme dosage $Y_{E_{app}}$ was then calculated as the increase compared to the actual dosage, $Y_{E_{app}} = E_{app}/E - 1$.

$$Y = \frac{abE}{bE + 1} \quad (1)$$

The effect of PEG on cellulase stability and substrate hydrolyzability was studied in varying conditions, with an enzyme dosage of 4 FPU g^{-1} DM unless stated otherwise. Hydrothermally treated

wheat straw and Whatman 1 filter paper were hydrolyzed with or without PEG addition (20 mg g^{-1} substrate DM) at 50 °C, and at 200 rpm in 250 ml Erlenmeyer flasks in a total volume of 30 ml. Denaturing conditions for filter paper hydrolysis were achieved using baffled 500 ml Erlenmeyer flasks at 250 rpm at 60 °C in a total volume of 100 ml. Samples were taken after 24 h, 48 h and 72 h hydrolysis and analyzed for sugars by HPLC.

2.4 Analysis of sugars and PEG

Monosaccharides were analyzed with high-performance liquid chromatography (HPLC). The system comprised a Micro-Guard De-Ash pre-column (Bio-Rad, USA) and an SPO810 column (Shodex) coupled to a refractive index detector (Shimadzu). Deionized water at a flow rate of 0.7 mL min^{-1} was used as an eluent at 60 °C. Monosaccharides were identified and quantified based on their retention times and peak areas relative to an external standard calibration.

Soluble PEG was determined with the 1260 Infinity HPSEC system (Agilent, Germany), described previously in quantitative lignin analysis [30]. For analysis of PEG, 0.1 M sodium phosphate buffer (pH 5) was used to elute a 6 mm x 40 mm Ultrahydrogel® guard column connected to a series of three (500 Å, 250 Å, and 120 Å) 7.8 mm x 300 mm Ultrahydrogel® size-exclusion columns (Waters, USA) at a flow rate of 0.5 mL min^{-1} and at 30 °C. The refractive index detector was calibrated as a concentration detector, and PEG 4000 was identified according to its retention time of 48.0 min.

2.5 Analysis of phenolics, cellulose surface area and water retention

Water-soluble phenolic compounds in the hydrolysates were determined according to the Folin–Ciocalteau method against gallic acid standards [31]. The adsorption of Congo Red (Direct red 28, Merck) on cellulose was studied according to Wiman et al., (2012) [32] with or without the addition of 20 mg g^{-1} PEG. HT-straw (50 mg DM) was incubated with 4 ml of Congo Red solution at concentrations of 2, 1, 0.5, 0.25 and 0.125 g L^{-1} in 30 mM phosphate buffer, pH 6.0 in capped glass tubes overnight at 60 °C on a shaker at 200 rpm in a tilted position. After incubation, the tubes were centrifuged, the supernatant was filtered through a 0.45 µm PFTE-filter to remove fine particles and

the Congo Red remaining in solution was determined spectrophotometrically at 498 nm. For determining the effect of PEG on the water retention value (WRV), 1.54 g DM of HT-straw was incubated for 2 h at 50 ºC in 50 mM phosphate buffer, pH 5.0 at 5% solids concentration with or without 20 mg g^{-1} PEG. The suspension was poured into a cylinder with a steel net on the bottom and centrifuged at 3000 RCF (Relative Centrifugal Force) for 15 min. The remaining water per dry matter was determined by drying overnight at 105 ºC. The procedure was performed in duplicate.

3. RESULTS AND DISCUSSION

3.1 Effect of PEG on apparent enzyme dosage

The effect of five different PEG dosages (2 – 50 mg g^{-1} substrate DM) on hydrolysis yield was determined at three different enzyme dosages. Without PEG-addition, the enzyme dosages of 2, 4 and 8 FPU g^{-1} (per substrate DM) led to the hydrolysis of 19.0, 29.8 and 47.1% of carbohydrates, whereas the largest PEG-addition of 50 mg g^{-1} DM increased hydrolysis to 28.5, 43.3 and 66.3%, respectively (Fig. 1A). At all enzyme dosages, close to maximal benefit was reached with a PEG addition of 20 mg g^{-1} DM, with little further increase at 50 mg g^{-1}. This is in accordance with the frequently reported optimum PEG dosage of 20–25 mg g^{-1} DM for pretreated lignin-containing agricultural residues [4,12,19].

Hydrolysis yields were compared to a hydrolysis standard (Fig. 1B) and converted into apparent enzyme dosages, i.e. the dosages required for reaching equal hydrolysis yields without PEG-addition. The hydrolysis standard (Fig. 1B) showed typical asymptotic behaviour of cellulose hydrolysis, where the benefit of further enzyme addition decreases as a function of hydrolysis degree [18,33]. The %-increase in the apparent enzyme dosage resulting from PEG addition, denoted as $Y_{E_{app}}$, is presented in Fig. 1C, as it proved to be particularly important for the findings in this work. It was observed that $Y_{E_{app}}$ was similar at all enzyme dosages, except for the 20 and 50 mg g^{-1} PEG loadings at 8 FPU g^{-1} enzyme dosage, where the effect of PEG addition was pronounced. At a PEG dosage of 10 mg g^{-1}, $Y_{E_{app}}$ was 53% ± 5.8% (standard error) with all enzyme dosages. The highest PEG dosage of 50 mg g^{-1} further increased $Y_{E_{app}}$ to 81% ± 4.0% with enzyme dosages 2 and 4 FPU g^{-1}.

However, $Y_{E_{app}}$ at the highest enzyme dosage of 8 FPU g^{-1} was as high as 173%, corresponding to an increase in hydrolysis yield from 47.1% to 66.3%, which would have required an enzyme dosage of 21.9 FPU g^{-1} without PEG. Considerably smaller increases in enzyme activity by PEG (up to only 18%) have previously been calculated [34], when the non-linear dosage response of hydrolysis has not been accounted for. This inevitably leads to underestimation, since the proportional increase in yield is always smaller than the corresponding increase in enzyme dosage, underlining the importance of managing the non-linearities in lignocellulose hydrolysis.

As expected, it is clear that there is no constant factor for converting PEG into an equivalent enzyme amount, and instead, the enzyme equivalent of PEG addition depends on both enzyme dosage and PEG loading. However, the similar $Y_{E_{app}}$ at most conditions provided an important lead for elucidating the relationship. The next task was to compare this effect to adsorption of PEG on the substrate.

3.2 Role of adsorption of PEG

The interactions of PEG with lignocellulose materials were characterized by determining adsorption of PEG on HT-straw, wheat straw lignin (Green Value lignin), acetylated wheat straw lignin and cellulose filter paper. Adsorption A (mg g^{-1}) of PEG on HT-straw and straw lignin was found to follow the Langmuir adsorption isotherm (Eq. 2), which by definition indicates reversible adsorption (Fig. 2A) in equilibrium with the soluble PEG concentration c_{eq} (g L^{-1}), with an equilibrium constant K (L g^{-1}).

$$A = A_{max}\frac{Kc_{eq}}{Kc_{eq}+1} \qquad (2)$$

This confirmed Langmuirian adsorption of PEG also on isolated wheat straw lignin, previously only observed for pretreated biomasses [5,12,19]. No adsorption could be determined on filter paper or acetylated lignin, where the phenolic and aliphatic hydroxyls have been covalently blocked, indicating that PEG was exclusively adsorbed on lignin and the adsorption was dependent on the hydroxyl groups of lignin. This is in accordance with the role of phenolic hydroxyls of lignin in PEG adsorption

suggested by Börjesson *et al.* [11] and Sipos *et al.* [7]. According to the isotherms, the maximum PEG-adsorption capacity A_{max} of HT-straw was 9.5 mg g^{-1} (Table 1). Straw lignin had a ten-fold maximum adsorption capacity compared to HT-straw, and three-fold compared to the lignin content of HT-straw. This suggests that in addition to hydroxyl groups, accessible surface area of lignin governs the extent of adsorption of PEG on lignin.

Table 1. The maximum adsorption capacity A_{max} and the equilibrium constant K for Langmuir isotherms for PEG adsorption on fresh and hydrolyzed HT-straw and straw lignin.

	A_{max}, mg g^{-1}	K, L g^{-1}	R^2
HT-straw	9.5	12.8	0.976
HT-straw, 2 FPU g^{-1}	10.7	15.3	1.000
HT-straw, 4 FPU g^{-1}	11.2	16.3	1.000
HT-straw, 8 FPU g^{-1}	12.5	16.0	0.999
Straw Lignin	94.1	29.6	0.97

The plateau in adsorption of PEG was reached above an initial dosage of 20 mg g^{-1}, which coincided with the plateauing effect on hydrolysis as shown above. In order to study the relationship between adsorption of PEG and hydrolysis yield, the concentration of soluble PEG was determined from the hydrolysates described above, and the corresponding adsorption was calculated as per mg g^{-1} initial substrate DM. It was found that the %-increase in apparent enzyme dosage ($Y_{E_{app}}$) correlates linearly with the amount of adsorbed PEG (Fig. 2B; Eq. 3). This rule was broken only by the 20 and 50 mg g^{-1} PEG loadings with 8 FPU g^{-1} enzyme dosage, which represented the high end of hydrolysis yield and PEG adsorption, and showed a higher effect than expected from the trend. For the range covering PEG adsorption of 0–10.8 mg g^{-1}, $Y_{E_{app}}$ followed adsorption with a slope of $\beta = 0.072$ g mg^{-1} ($R^2 = 0.94$).

$$Y_{E_{app}} = \beta A \quad (3)$$

It has been shown that cellulases are not only adsorbed on lignin, but are also inactivated in contact with lignin [14]. The rate of interactions between enzymes and solid lignin could be expected to be proportional to the accessible lignin surface area, which is decreased by adsorption of PEG. This is the most straightforward explanation for the observed linearity of the effect of PEG adsorption on $Y_{E_{app}}$. It can also be generalized that first order reaction kinetics lead to an equal proportional change in constant time, regardless of initial concentration. This means that an equal percentage of cellulases is inactivated on lignin surface regardless of enzyme dosage, if the inactivation follows pseudo-first order kinetics. Accordingly, partial prevention of the inactivation would also lead to equal proportional improvement at any enzyme dosage. This is exactly what was observed as the $Y_{E_{app}}$ did not depend on enzyme dosage in the linear range. This implies that the lignin-blocking effect of PEG on hydrolysis may therefore be pinpointed as prevention of pseudo-first order inactivation of cellulases on lignin, which serves as a possible kinetic basis for the linearity and proportionality observations of $Y_{E_{app}}$. However, this behaviour would not fit to alternative second order adsorption kinetics of cellulases or competitive adsorption of PEG and enzymes on lignin, both of which could be considered possible. Further research is therefore needed for directly determining the kinetics of cellulase inactivation by lignin. In any case, this finding illustrates the direct link between PEG adsorption and enzyme inactivation on lignin and for the first time, provides a direct link between PEG adsorption kinetics and enzyme consumption.

In order to determine the changes in PEG adsorption kinetics in the course of hydrolysis, Langmuir isotherms were fitted for PEG adsorption observed in the hydrolysates as mg g^{-1} per initial DM (Fig. 2C). A separate isotherm was fitted for each enzyme dosage, representing different degrees of hydrolysis. The adsorption parameters K and A_{max} were then determined and plotted against the corresponding maximum hydrolysis yield (Fig. 2D). It was observed that the maximum adsorption capacity was increased linearly as a function of hydrolysis degree, but the increase was only 30% of the initial, in accordance with a previous report of a small increase in PEG adsorption in the course of hydrolysis [5]. This indicates that the lignin surface accessible to PEG is only slightly increased by the hydrolysis of carbohydrates, in agreement with a previous conclusion by the authors, when only minor

changes in surface accessible phenolic hydroxyls of lignin were observed during the hydrolysis of HT-straw [35]. Interestingly, according to the changes in the kinetic parameters during hydrolysis, there was no evidence of the enzyme dosage affecting PEG adsorption in a competitive manner, although the dosages were at a mass range comparable to the PEG dosages (2–8 FPU g^{-1} DM corresponding to protein amounts of 1.7–6.7 mg g^{-1} DM). Competitive adsorption of an increasing amount of enzymes should have led to a decrease in the observed K in PEG adsorption. Instead, the contrary was observed as K was higher after hydrolysis than in the absence of enzymes (Fig. 2D). In conclusion, adsorption of PEG decreases adsorption of cellulases on lignin [5,7,8,11], but not *vice versa*. This indicates that the effect of PEG on enzyme-lignin interactions is more complicated than direct competition for surface area. Perhaps the linear PEG-molecules are intertwined with the lignin network, decreasing lignin mobility and therefore constraining the extent of interaction between lignin molecules and cellulases. This suggests that the effects of PEG on the physical structure of lignin could be a revealing subject for research in the future.

Divergence of $Y_{E_{app}}$ from the linear trend at 8 FPU g^{-1} with high PEG dosages suggests that the effect of PEG is boosted by an additional mechanism at high hydrolysis degrees, allowing hydrolysis of the least hydrolysable parts of pretreated straw. This could result from a direct effect on the substrate or the enzyme, leading to increased hydrolyzability or enzyme stability. These possibilities were further studied below.

3.3 Evaluation of Effects of PEG on enzyme stability and cellulose availability

An additional hydrolysis boost by PEG not explained by PEG adsorption was observed when both PEG dosage and hydrolysis degree were high. Several experiments were carried out in order to identify possible direct effects of PEG on enzyme stability or cellulose accessibility in HT-straw. In order to test the effect of PEG on enzyme stability during hydrolysis, filter paper was hydrolyzed at denaturing conditions at 60 ºC with severe agitation with or without a PEG addition of 20 mg g^{-1} and an enzyme dosage of 4 FPU g^{-1}. Denaturing conditions led to a 38–46% decrease in hydrolysis degree compared to normal agitation at 50 ºC, and the decrease was not reversed by the presence of PEG, which showed no effect at either conditions (Fig. 3A). Therefore no indication of enzyme stabilization

was observed and the lack of PEG effect with filter paper was in accordance with previous reports of low effect of PEG on delignified materials [3–8]. However, several reports have shown a beneficial effect of PEG in hydrolysis of microcrystalline cellulose (Avicel), indicating that PEG affects hydrolysis in additional ways which are not related to lignin [15,16]. Hsieh et al., [16] reported a small stabilizing effect of PEG on cellobiohydrolases in a pre-incubation experiment, but the role of stabilization was considered to be minor.

Next, it was tested whether an excess enzyme dosage of 60 FPU g^{-1} could overcome the benefit of PEG in the hydrolysis of HT-straw (Fig. 3A). After 24 h, PEG still showed a small positive effect on hydrolysis yield, but after 48 h the difference was no longer statistically significant ($p > 0.05$). This shows that although PEG has a particular effect in the hydrolysis of the most difficult parts of lignocellulose, it does not breach any barriers in substrate hydrolyzability that could not also be overcome by enzymes alone.

It was hypothesized that PEG could directly affect the substrate, improving hydrolyzability. This could be related to surfactant effects of PEG that remove lignin from cellulose surfaces, or increased water retention that could lead to increased porosity and enzyme accessibility. These ideas were studied further by determining the amount of phenolic compounds dissolved by PEG, and the effect of PEG on cellulose surface area and water retention capacity of HT-straw.

The amount of dissolved phenolic compounds was determined from the hydrolysates produced with the different PEG and enzyme dosages presented above. Dissolution of phenolic compounds correlated with hydrolysis degree (Fig. 3B), in accordance with previous results by the authors [35]. It was also found that the addition of PEG slightly increased the dissolution of phenolic compounds at PEG dosages of 10–20 mg g^{-1}. However, this increased dissolution of phenolic compounds was observed regardless of hydrolysis degree, and therefore did not show consistent relationship with the boost in PEG effect, which was only observed at a high hydrolysis degree.

It was hypothesized that PEG may function as a detergent, revealing cellulose surface by removing small lignin fragments from the solid fraction. For testing this hypothesis, the effect of 20 mg g^{-1} PEG addition on the cellulose surface area of HT-straw was studied by determining the isotherm of Congo Red adsorption on HT-straw, used for determining cellulose surface area in

biomass [32]. However, no effect of PEG was observed in Congo Red adsorption isotherms, indicating that dissolution of phenolics by PEG addition did not reveal fresh cellulose surfaces (Fig. 3C).

Finally, the effect of PEG (20 mg g^{-1}) on the water retention capacity of HT-straw was determined, but no effect was observed, as the water retention value was found to be 1.2 (±0.01) g g^{-1} in the absence or presence of PEG.

In conclusion, in spite of several attempts, no evidence was found on improvement of hydrolysis by a direct effect of PEG on enzyme stability or cellulose accessibility. However, an increased dissolution of phenolics shows that direct physicochemical changes do occur in the substrate at high PEG loadings.

3.4 Determination of the enzyme equivalent of PEG

In order to construct a model for the enzyme equivalent of PEG, the effect of PEG on apparent enzyme activity (Eq. 3) was combined with the adsorption kinetics of PEG. Analogously with the PEG adsorption projections by Vaidya *et al.* [19], the amount of adsorption of PEG can be predicted from the total PEG loading P and the substrate concentration c_{DM}. This allows determination of PEG adsorption at higher substrate concentrations, which are more relevant for industrial-scale hydrolysis [22,36]. The initial PEG dosage P (mg g^{-1} DM) equals the sum of adsorbed and free PEG per dry matter, according to Eq. 4.

$$P = \frac{Ac_{DM} + (1 - c_{DM})c_{eq}}{c_{DM}} \qquad (4)$$

Solving c_{eq} from the Langmuir equation and substituting the result in equation 4 allows solving adsorption A as the function of initial PEG dosage. This results in a quadratic equation, giving PEG adsorption as a function of initial PEG concentration and solids concentration (Eq. 5)

$$A(P, c_{DM}) = \frac{-b - \sqrt{b^2 - 4ac}}{2a} \qquad (5)$$

$$a = K, \qquad b = 1 - PK - A_{max}K - \frac{1}{c_{dm}}, \qquad c = PA_{max}K$$

To conclude, the enzyme equivalent E_{eqv} of PEG can be calculated with the Langmuir adsorption parameters of PEG and the slope β of the proportional PEG effect. Multiplying actual enzyme amount

E with its proportional increase $Y_{E_{app}}$ gives the result as mg g^{-1} DM. Therefore, by substituting equation 5 in equation 3, and multiplying by E, we arrive at the enzyme equivalent of PEG as a function of enzyme dosage, PEG loading and solids concentration (Eq. 6).

$$E_{eqv}(E, P, c_{DM}) = E\beta A(P, c_{DM}) \quad (6)$$

3.5 Model constraints and extrapolation

The model presented in section 3.4 represents the enzyme savings accounted for by adsorption of PEG on lignin, and does not account for the additional effects that were observed when both enzyme (8 FPU g^{-1}) and PEG ($\geqslant$20 mg g^{-1}) loadings were high. The model is expected to be applicable also to other lignocellulosic biomasses processed by hydrothermal or equivalent pretreatments, but it will need to be recalibrated casewise. However, since the experiments were carried out at 5% DM, projection of the model to higher solids concentrations requires elaboration. First of all, the model relies on Langmuir theory of adsorption, where the parameters are unaffected by solids concentration, as only the equilibrium will be shifted. Therefore projections of adsorption to higher DM are applicable on firm theoretical basis, and have also been presented previously [19]. The second question is whether the effect of the solids concentration on hydrolysis may affect the model parameters, namely E_{app} and subsequently, $Y_{E_{app}}$ and β. The effect of solids concentration on hydrolysis is often simplified as a negative linear correlation [37], and it persists also in the presence of PEG and other additives [10]. Based on the data presented in those two studies, the change in hydrolysis yield on lignin containing biomasses is -2.8 % (Standard error 0.3%, n = 8) from the yield at 5% DM per 1 % increase in DM, implying that the change between two solids concentrations can be simplified as a multiplication with a constant. It is observed that modifying the current hydrolysis results with a constant factor will not affect E_{app}, $Y_{E_{app}}$ or β, since a modified hydrolysis yield will correspond to the same apparent enzyme activity E_{app} on a similarly modified hydrolysis standard. In general, conversion of hydrolysis yields into corresponding enzyme amounts eliminates overall changes in absolute hydrolysis yield from the model, and consequently, the effect of dry matter

concentration on the model parameters is expected to be minor. This justifies extrapolation of the model to higher DM.

3.6 Cost-optimization of PEG addition

The benefits of PEG utilization were simulated and the optimal PEG dosage was determined as a function of solids loading and enzyme dosage, according to the parameters determined above for hydrothermally treated wheat straw. Since only the adsorbed PEG delivers the positive effect, the PEG remaining in solution is 'wasted'. Therefore the efficiency of PEG utilization is expected to increase with increasing solid to liquid ratio [19]. The percentage of adsorbed PEG was determined from equation 5 as a function of solids loading at PEG dosages from 5 to 20 mg g^{-1} (Fig 4A). The corresponding reduction in enzyme consumption, *i.e.* the percentage of the apparent enzyme dosage accounted for by the effect of PEG, is obtained from equation 7 and is presented in Fig 4B. For derivation of equations 7-9, see Appendix A.

$$E_{Saved} = \frac{Y_{E_{app}}}{Y_{E_{app}} + 1} \quad (7)$$

At a PEG dosage of 10 mg g^{-1}, the percentage of adsorption is 66% at 5% solids loading and 81% at 20% solids loading, corresponding to enzyme savings of 32% and 37% respectively. Previous estimates of enzyme savings by PEG addition range from 30% [21] up to 82% [19].

In order to find the optimal PEG dosage, the cost of PEG addition must be weighed against the corresponding reduction in enzyme costs. Estimates for on-site cellulase production costs vary from optimistic 2.7–4.8 \$ kg^{-1} [19,38,39] to 10 \$ kg^{-1} [40] enzyme protein, and above 10 \$ kg^{-1} for commercial enzymes. The price of PEG is expected to be lower, with an estimate of 1 \$ kg^{-1} [19]. Given the ambiguity of commercial price data, a more generalizable study can be made using the ratio of enzyme price to PEG price, denoted here as ϵ. PEG addition is profitable if the cost is lower than the cost of an equivalent enzyme amount. Accordingly, the PEG dosage can be increased as long as the marginal benefit of the increase is positive. In other words, the difference of the cost of the increase dP and the cost of the equivalent enzyme addition $dE_{eqv}(P)$, must be positive. Finally, the

optimum PEG dosage P_{opt} is reached when the marginal benefit reaches zero, which can be expressed with the price-ratio ϵ as follows:

$$dP - \epsilon * dE_{eqv}(P) = 0 \qquad (8),$$

This can be rearranged into a derivative of $Y_{E_{app}}$ with respect to P.

$$\frac{1}{\epsilon} = E * \frac{dY_{E_{app}}(P)}{dP}\bigg|_{P=P_{opt}} \qquad (9)$$

The optimal PEG dosage P_{opt} was solved iteratively from equation 9 at price ratios of 2, 5 and 20 for different solids loadings as a function of enzyme dosage (Fig 4C). A useful property of the model results from the proportionality of the PEG effect, as the enzyme unit (g^{-1}) does not need to be explicitly defined and can be chosen freely (e.g. mg g^{-1}, FPU g^{-1}, ml g^{-1}), as long as the enzyme price is determined in the same unit. For example, the optimum PEG dosage at an enzyme dosage of 10 mg g^{-1} and solids concentration of 10% is 6.6 mg g^{-1}, 11.4 mg g^{-1} and 14.2 mg g^{-1} for price-ratios of 2, 5 and 10, respectively. This indicates that the optimum PEG dosage can be lower than the dosage where maximal effect of PEG is reached (~20 mg g^{-1} [4,12,19].), especially at low enzyme dosages. The optimum PEG dosage increases with increasing enzyme dosage as well as with increasing price ratio, as higher PEG dosages become more affordable compared to enzymes. Solids loading had a smaller effect on the optimum PEG dosage, showing higher optima for high DM at small enzyme dosages, reflecting the higher adsorption efficiency. Increasing enzyme dosage leads to an intersection point, where the optimum is equal for all solids loadings. At higher enzyme dosages, a very high PEG addition becomes feasible, since even a minor increase in $Y_{E_{app}}$ corresponds to a large amount of enzymes. Therefore, higher optima are observed for lower DM, as a higher PEG dosage is required for saturation of adsorption.

It is also observed that PEG addition is not feasible at all at smallest enzyme dosages, as the improvement corresponds to a very small enzyme amount. At price-ratios of 2, 5 and 20, PEG addition becomes feasible at enzyme dosages of 7.5, 3.0, and 1.5 g^{-1} respectively at 10% DM. To study this observation more closely, a threshold ϵ was determined, above which PEG addition becomes

profitable. This threshold is found where the optimum PEG dosage is zero *i.e.* by setting $P = P_{opt} = 0$ in equation 9. ϵ is inversely proportional to E, and thus gives a hyperbolic curve, scaled by the derivative (Fig. 4D). The curve first quickly descends as a function of enzyme dosage up to a turning point (vertex) at a dosage of 3.9 g^{-1}. At an enzyme dosage of 2 g^{-1}, the enzymes should be 7.5 times more expensive than PEG, before PEG addition should be considered. At enzyme dosages of 5 g^{-1} and 10 g^{-1}, PEG addition is profitable at considerably lower price ratios of 3 and 1.5, respectively. The effect of solids concentration on the threshold ϵ was almost negligible.

The model presented in this study allows optimization of PEG-dosage as an isolated variable cost component of lignocellulosic sugar production, and it could be combined with a more general hydrolysis response model, as a modifier of the effective enzyme dosage. Broader optimization of enzymatic hydrolysis conditions by minimizing sugar production costs requires integration of the model into a techno-economic analysis of a lignocellulosic sugar production process, which is a subject for future research.

4. CONCLUSIONS

Two novel observations were made that provided a crucial link between adsorption kinetics of PEG and the effect of PEG on enzyme consumption in lignocellulose hydrolysis. It was found that the addition of PEG in enzymatic hydrolysis of hydrothermally treated wheat straw leads to a constant proportional increase in apparent enzyme dosage, regardless of the absolute enzyme dosage, and that the increase correlates linearly with the adsorbed amount of PEG. Kinetic basis for these observations was proposed as the observations support pseudo-first order denaturation kinetics of cellulases upon contact with on lignin, and prevention thereof by adsorption of PEG. These observations allowed modelling the effect of PEG as an equivalent enzyme addition. Model constraints and justification for model projections were presented, and the model was applied for the first cost-optimization of PEG dosage by analysis of the marginal benefit against marginal cost of PEG addition. It was concluded that PEG addition improves the process economy if a threshold price-ratio is exceeded, *i.e.* if enzyme price is sufficiently higher compared to the price of PEG, and this threshold is lower for higher

enzyme dosages. Above the threshold, the optimum PEG dosage was presented for various enzyme dosages, solids concentrations and price-ratios.

The findings presented in this study provide novel tools for case-by-case evaluation of PEG-addition, which is an economically viable route for improving lignocellulose hydrolysis when enzyme consumption and cost are sufficiently high.

Declaration of competing interests

The authors declare that no competing interests exist regarding this work.

CRediT author contributions statement

Ville Pihlajaniemi: Conceptualization, investigation, methodology, software, formal analysis, writing – initial draft, **Anne Kallioinen:** Investigation, Methodology, writing – review & editing, **Mika Sipponen:** Conceptualization, investigation, Methodology, writing – Review & editing, **Antti Nyyssölä:** Supervision, writing – Review & editing.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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APPENDIX A. SUPPLEMENTARY INFORMATION

Supplementary information can be found in online version of this article.

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Figure captions

Figure 1. Effect of PEG on hydrolysis. A) Hydrolysis yield from HT-straw as a function of the dosages of enzyme and PEG. B) Non-linear hydrolysis standard with 1 – 20 FPU g^{-1}. C) Increase (%) in the apparent enzyme dosage by PEG-addition. The dots represent actual data points and the surfaces are produced by cubic interpolation of the averages.

Figure 2. Adsorption studies of PEG. A) Adsorption of PEG on different materials. B) Increase of apparent enzyme dosage as a function of adsorbed PEG. Trendline (dashed line) excluding two divergent points (upper right corner). C) Adsorption of PEG after hydrolysis with different enzyme dosages. D) The adsorption capacity and Langmuir coefficient as a function of hydrolysis degree. Error bars represent ± standard error.

Figure 3. A) Hydrolysis yield from filter paper (FP) with (+) or without (-) PEG under denaturing conditions (60 ºC and severe agitation) or from HT-straw with an excess enzyme dosage. B) Dissolution of phenolics by PEG. C) Effect of PEG on CongoRed adsorption of HT-straw. Error bars represent ± standard error.

Figure 4. Simulation of the effects of PEG on enzyme consumption. A) The percentage of adsorption from the total PEG dosage as a function of dry matter concentration in hydrolysis at different PEG dosages, indicated as mg g^{-1} on each plot. B) The corresponding savings in enzyme consumption. C) Optimal PEG dosage at different ratios ϵ of enzyme price to PEG price, and at different dry matter concentrations, as a function of enzyme dosage per g biomass DM. The unit of the enzyme dosage can be chosen freely (e.g. FPU g^{-1} or mg g^{-1}), as long as enzyme price is defined in the same units. D) Threshold ϵ, above which PEG addition becomes profitable.

Figure 1.

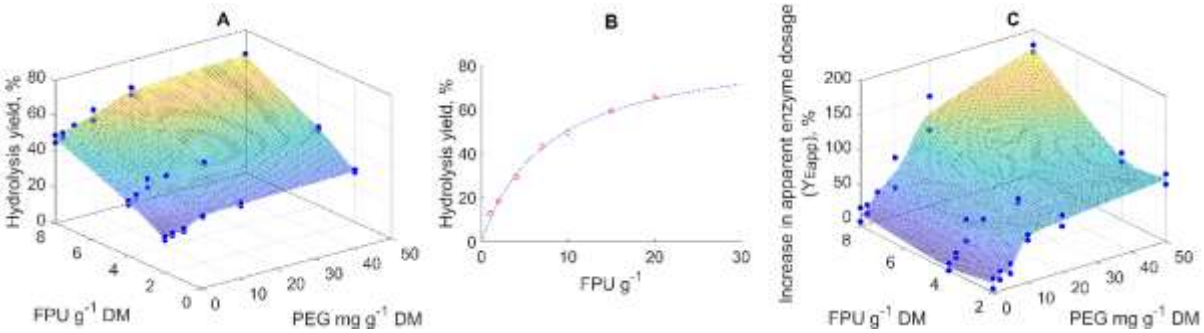

Figure 2.

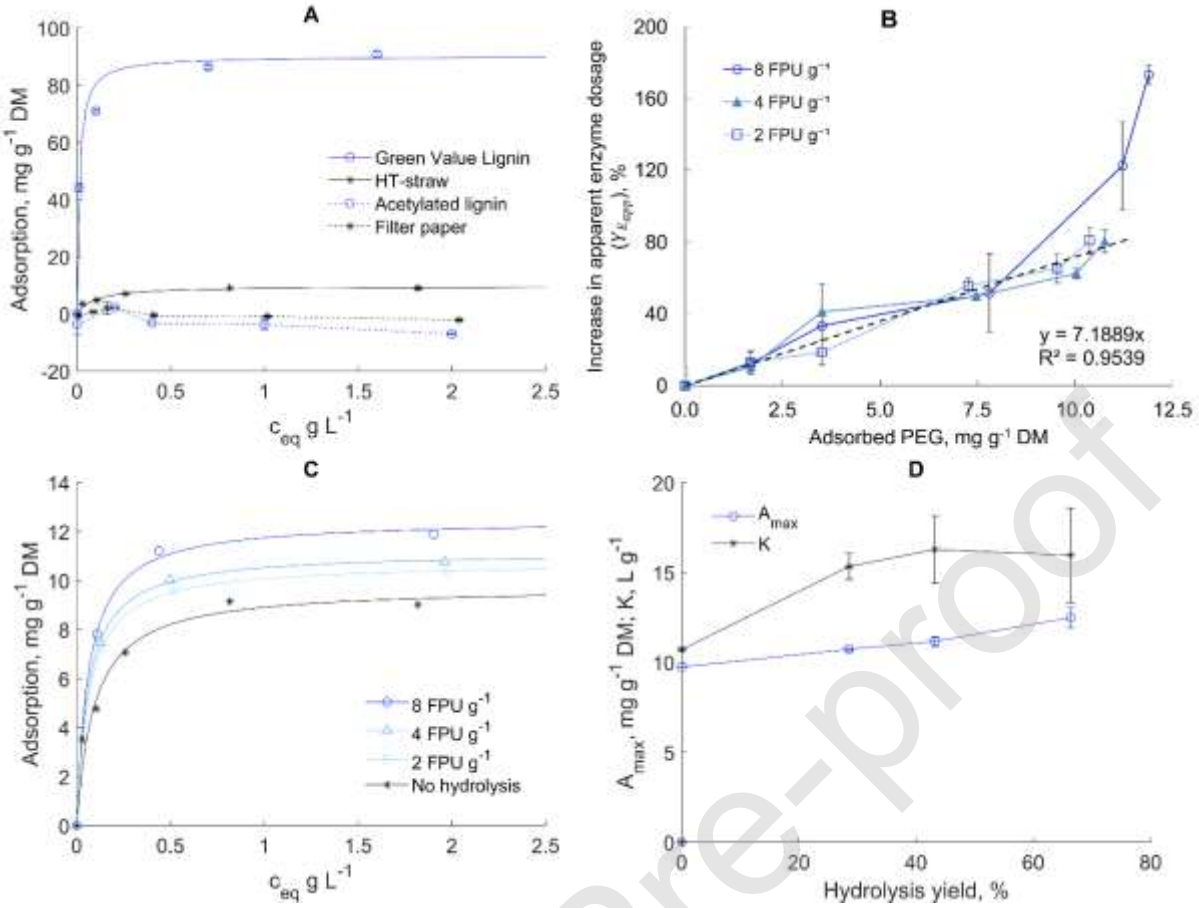

Figure 3.

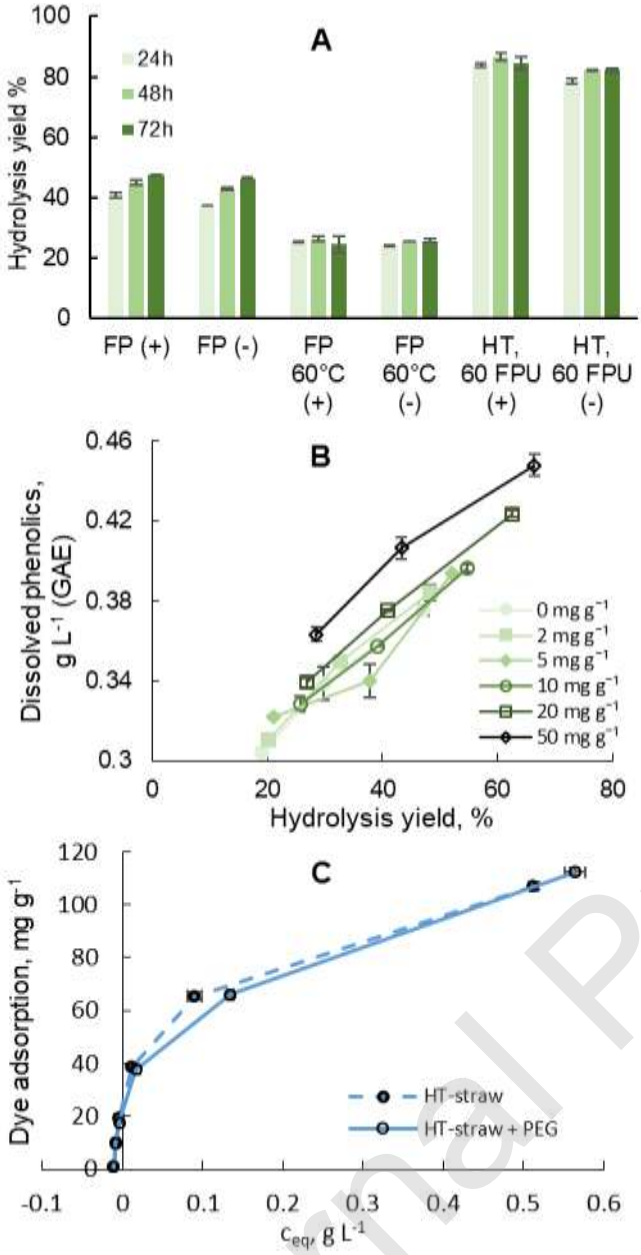

Figure 4.