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Enhanced enzymatic conversion of softwood lignocellulose by poly(ethylene glycol) addition

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Abstract

Ethanol production from lignocellulose has great potential and is an important step in changing fuel consumption to a more environmentally friendly alternative. Lignocellulose is a large source of biomass. However, with lignocellulose and softwood lingocellulose in particular, high conversion of cellulose into fermentable sugars requires large amounts of enzymes. Addition of surfactants is known to increase the enzymatic conversion and decrease the amount of enzymes needed. Surfactants and polymers with various amount of ethylene oxide (EO) content were used to study the conversion of steam-pretreated spruce lignocellulose. Increasing conversion was obtained with longer EO chains on the non-ionic surfactants. Similar results were obtained by using only the hydrophilic part of the surfactant, i.e. by addition of ethylene oxide polymers such as poly(ethylene glycol) (PEG) to the hydrolysis mixture. Interactions of enzymes and PEG with substrate was monitored with ^{14}C-labeled PEG 4000 and ^{3}H-labeled Cel7A (CBH I), the dominating cellulase from *Trichoderma reesei*. Addition of PEG to enzyme hydrolysis of lignocellulose increased the conversion from 42% without addition to 78% in 16 h. Adsorption of Cel7A decreased from 81 to 59%. No effect of PEG was seen on a delignified substrate. By addition of PEG it was possible to perform hydrolysis at 50 °C leading to both high cellulose conversion (80%) and shorter process time (48 h). Two different interactions are proposed in PEG adsorption on lignocellulose, hydrogen bonding and hydrophobic interactions. Our conclusions from experiments on lignocellulose and delignified substrate are that EO containing surfactants and polymers, such as PEG, bind to lignin by hydrophobic interaction and hydrogen bonding and reduce the unproductive binding of enzymes.

Keywords: Cellulase; Cellulose; Lignocellulose; Lignin; Surfactants; PEG; *Trichoderma reesei*; Adsorption; Cel7A; Enzymatic hydrolysis

1. Introduction

There is currently a great interest in developing processes for production of alternative fuels. Enzymatic hydrolysis of cellulose into soluble sugars for production of ethanol as liquid fuel has been studied extensively. There are, however, still obstacles for introduction of a large scale process based on enzymes. The rate of hydrolysis decreases fast over time resulting in long process times to reach sufficient conversion. To overcome this, large amount of enzymes are needed, resulting in high process cost. Spruce is a potential important source for bioethanol production due to its abundance in the northern countries. Softwood substrates have been shown to be more difficult to hydrolyze compared to hardwood substrates, these can with less effort be hydrolyzed to a level of 90–95% [1]. The rate limiting step in bioethanol production from wood raw materials using enzymes is the hydrolysis, which is true for both separate hydrolysis and fermentation or simultaneous saccharification and fermentation. Many studies have been presented over the years aiming to understand and affect the inhibiting factors in enzymatic hydrolysis of lignocellulose substrates. Several reasons for low yield in enzymatic conversion have been presented: reduced accessible surface area of the cellulose in lignocellulose complex leading to restricted access for enzymes [2]; restricted pore volume of the substrate [2,3]; slow enzyme kinetics for crystalline cellulose [4] and obstacles in the structure of cellulose leading to unproductive enzyme binding [5,6]. Lignin has also been identified to have a high binding affinity for cellulase proteins [7,8]. Both addition of lignin [9] as well as the content of lignin [10] has been shown to be responsible for inhibitory effects on the degradation of cellulose. It was recently found that cellulases lacking cellulose binding module (CBM) also have a high affinity for lignin, indicating the presence of lignin-binding sites on the catalytic module [8].

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Surfactants have been shown to enhance enzymatic conversion of lignocellulose substrates. Kaar and Holtzapple showed that addition of Tween to hydrolysis of corn stover lowered the kinetic constants related to substrate affinity and enzyme binding sites on the substrate in a way that helps the enzymes maintain affinity for the substrate [11]. Balesteros et al. showed that addition of Tween 80 increased the conversion of steam exploded poplar by modifying the adsorption and desorption of enzymes [12]. In previous work we could conclude that surfactants adsorb to lignin and prevents unproductive adsorption of enzymes [13]. The binding of enzymes to lignin can be reversed by addition of surfactants and polymers that interact with the substrate [9,13]. Addition of surfactants has also been described to have a stabilizing effect on enzymes. Kaar and Holtzapple suggest that Tween can protect enzymes from thermal denaturation in hydrolysis conducted at higher temperatures [11].

The research prior to the work presented here has focused on understanding the mechanism behind the effect of surfactant additions to enzymatic hydrolysis of lignocellulose [13]. In that work non-ionic surfactants were found to have the best characteristics for obtaining the fastest and highest conversions. Several possible explanations for the positive effect of non-ionic surfactants were studied in order to reveal the mechanism. The added surfactant did not stabilize the enzymes against denaturation at the temperature used (40 °C). It was concluded that the presence of lignin was important to achieve an increased conversion with addition of surfactant. No increase in activity was found on pure cellulose or soluble substrates. By mimicking the surfactant effect by adding BSA which is known to reduce unspecific adsorption [14] we could suggest that the major obstacle in enzymatic hydrolysis of lignocellulose was the adsorption of significant amounts of enzymes on exposed lignin surfaces.

The additives often shown to be most effective on lignocellulose substrates are non-ionic surfactants or polymers containing ethylene oxide (EO) [9,11–13,15]. We can now show that ethylene oxide polymers such as poly(ethylene glycol) (PEG) has similar or even stronger effect. Understanding the interactions between additives, enzymes and lignin is important in order to make changes in the process concerning choice of enzymes, pretreatment of raw material and process conditions. The present study is focused on the enzymatic hydrolysis of the softwood substrate spruce lignocellulose and aims to give further understanding of the mechanism behind the enhancing effect on the conversion by addition of ethylene oxide based surfactants and polymers. For development of the enzymatic ethanol process we have studied the possibility with PEG addition to use higher temperature and shorter process time in the hydrolysis step.

2. Materials and methods

2.1. Substrates

Steam-pretreated spruce (SPS) was used as a substrate in all experiments and was a gift from Prof. Guido Zacchi, Department of Chemical Engineering, Lund University. SPS was produced from spruce chips. The chips were impregnated with 3% (w/w water content in the spruce chips) SO_2. The chips were then pre-treated for 3–5 min at 210–215 °C in saturated steam. The pre-treatment method is further described by Stenberg et al. [16]. After pre-treatment, the SPS substrate was washed with water to remove soluble material. The cellulose content was determined by a method provided by National Renewable Energy Laboratory/Midwest Research Institute (NREL/MRI) (Laboratory Analytical Procedure, LAP-002) [17]. Samples of SPS were weighed and dried at 40 °C for 3 days. The dried samples were treated with 3 ml of 72% H_2SO_4 and placed in water bath with a temperature of 30 °C. The samples were vortexed for 30 s every hour for 2 h. The samples were transferred to 100-ml autoclave bottles and diluted with 84 ml of Milli-Q water to give a H_2SO_4 concentration of 2.5%. The samples were autoclaved for 1 h at 121 °C. After 20 min of cooling, 20 ml of the samples were transferred to 50-ml bottles and neutralised with $CaCO_3$ to a pH between 5 and 6. Glucose concentration was analysed with a DX 500 High Performance Anion Exchange Chromatography system with Pulsed Amperometric Detection, HPAEC-PAD (Dionex, Sunnyvale, CA) using a CarboPac™ PA100 analytical column (Dionex) under isocratic conditions (100 mM NaOH). Cellulose content of SPS was found to be 51.1 ± 1.0% using three replicates. The content of dry material in SPS was 23.4 ± 0.1%. Delignified SPS was produced as described by Eriksson et al. [13].

2.2. Surfactants and polymers

The surfactants used in these experiments were all a gift from Akzo Nobel (Stenungsund, Sweden). Poly(ethylene glycol) (PEG) with molecular mass of 2000, 4000, 6000 and 8000 was obtained from Merck (Hochenbrunn, Germany). ^{14}C PEG 4000 was obtained from Amersham Biosciences (Uppsala, Sweden). The composition of used surfactants and polymers is shown in Table 1.

2.3. Enzymes

Hydrolysis experiments were performed with the commercial enzyme solutions Celluclast 1.51 which is produced by fermentation of a selected strain of *Trichoderma reesei* (now named *Hypocrea jecorina*) and Novozym 188 (Novozymes A/S, Bagsvaerd, Denmark) that is produced by *Aspergillus niger*. Celluclast 1.51 had cellulase activity of 86 FPU g^{-1} (filter paper units per gram of enzyme solution) and β-glucosidase activity of 33 IU g^{-1} (1 IU is defined as the amount of enzyme that produces 1 μM product per minute). Novozym had a β-glucosidase activity of 350 IU g^{-1}. The purified *T. reesei* cellobiohydrolase, Cel7A (formerly known as CBHI) was a gift from Dr. Maija Tenkanen, Department of the Applied Chemistry and Microbiology, University of Helsinki, Finland. Cel7A was purified as described elsewhere [18]. The purity of the Cel7A fraction was confirmed by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS–PAGE) stained with Coomassie brilliant blue.

2.4. Hydrolysis experiment

Hydrolysis experiments were performed in 1 ml of 50 mM sodium acetate at pH 4.8 using 1.8-ml screw-cap tubes. Hydrolysis was performed at 40 or 50 °C

Table 1
Chemical composition of surfactants and polymers

Surfactant/polymer	Composition
Alcohol ethoxylate (alkyl polyoxyethylene ethers)	CH_3–$(CH_2)_{8–10}$–O–$(CH_2–CH_2–O)_{4, 6, 8}$, CH_3–$(CH_2)_{15–21}$–O–$(CH_2–CH_2–O)_{80}$
Ricin oil ethoxylate[a] (Ricin oil polyoxyethylene ethers)	Ricin oil–O–$(CH_2–CH_2–O)_{28, 40, 75 \text{ and } 200}$
Poly(ethylene glycol) (molecular masses: 2000, 4000, 6000 and 8000)	HO–$(CH_2–CH_2–O)_n$–H ($\bar{n}$ = 45, 91, 136, and 182)

[a] Ricin oil ethoxylate is produced from castor oil that is composed of 90% unsaturated (C:18) ricinoleic fatty acid.

in a thermostated water bath. The tubes were inverted 20 times per minute. The reactions were terminated by filtering through a 0.22-μm syringe filter (Millipore, Bedford, MA). All experiments were performed in triplicate and mean values and standard deviations are presented. To describe significant differences between mean values throughout this article, *t*-test has been used. For each test the hypothesis of two mean values being equal has been tested at a level of significance for α equal to 0.05. A significant difference between two mean values are therefore indicated as ($P \leq 0.05$).

2.5. Sugar analysis

Glucose concentration in the filtrates from the hydrolytic experiments was analysed in a HPAEC-PAD chromatography system using a CarboPac^{TM} PA100 analytical column (see Section 2.1 for full details). An isocratic method with 100 mM NaOH was used for elution. The conversion expressed in percent was calculated from the sum of produced glucose and based on cellulose content in the substrate.

2.6. Cel7A adsorption

Cel7A adsorption was measured by adding tritium labeled Cel7A to concentrations between 0.01 and 0.1 μM in the enzyme mixture. Enzyme mixture containing the labeled Cel7A without addition of substrate was used to produce calibration curves for each experiment. Labeled Cel7A was quantified radiometrically (Beckman liquid scintillator LS 1801, Beckman Instruments, CA). Sample (100 μl) of filtrated (0.22 μm) supernatant was added to 7 ml Beckman ReadySafe scintillation cocktail and mixed extensively by vortex. Labelling of Cel7A was performed as described by Tack et al. [19]. The tritium labeled enzyme was compared with the unlabeled enzyme both in terms of hydrolytic as well as adsorption properties. There was no significant difference between the labeled and the unlabeled enzyme [13]. All experiments were made in triplicates.

2.7. Adsorption of poly(ethylene glycol)

PEG 4000 adsorption was measured by adding ^{14}C labeled PEG 4000 to the PEG solution in the adsorption experiment. Final labeled PEG concentration was 0.25 μM. After incubation and filtration the amount of unadsorbed PEG 4000 was measured radiometrically (Beckman liquid scintillator LS 1801, Beckman Instruments, CA). Sample (100 μl) of filtrated (0.22 μm) supernatant was added to 7 ml of Beckman ReadySafe scintillation cocktail. All experiments were made in triplicates.

Experiments for adsorption isotherm determination and time course for adsorption of PEG on SPS was carried out in a 1-ml reaction volume using 1.8-ml screw cap tubes. Both experiments were performed in 50 mM sodium acetate buffer at pH 4.8 and at a temperature of 40 °C.

3. Results

3.1. Surfactants with increased EO chain length

Non-ionic surfactants with ethylene oxide chains were analysed for their ability to enhance enzymatic hydrolysis of SPS. Cellulose conversion was analysed by quantification of soluble sugars with an HPAEC-PAD chromatography system. The complete enzyme system of *T. reesei* was used and the end product was glucose due to addition of β-glucosidase. Surfactants were compared in a 16 h enzymatic hydrolysis by adding 2.5 g l^{-1} surfactant (Fig. 1a and b). The general formula for the surfactants was $C_x(EO)_y$. Used surfactants had ethylene oxide chains of 4, 6, 8 and 80 EO units as hydrophilic part and linear alkyl chains of 9–11 and 16–22 methyl groups as hydrophobic part. No addition of surfactant resulted in a conversion of 54%. Addition of surfactant showed a significant ($P \leq 0.05$) increase in conversion, as previously observed [13]. However, increased enzymatic conversion of SPS was obtained when the length of the hydrophilic EO chain was increased and the hydrophobic alkyl chain of 9–11 methyl groups was kept constant. Conversion was even more enhanced when the number of EO units was increased to 80, in effect when the surfactant was transformed to an ethylene oxide polymer with an attached alkyl group. The adsorption of Cel7A (CBHI), which is the dominating cellulase in the *T. reesei* enzyme system, was significantly decreased with addition of surfactants ($P \leq 0.05$) and a tendency of decreased adsorption is seen with the length of EO chain (Fig. 1b).

3.2. Ethoxylated ricin oil surfactants with increasing EO chain length

Four surfactants based on ethoxylated ricin oil with ethylene oxide chain length of 28, 40, 75 and 200 EO units were added to further analyse the effect of ethylene oxide chain length when having a constant length of the hydrophobic part (Fig. 2). All surfactants increased the hydrolysis compared to no addition, resulting in conversions from 74 to 85% in 16 h. Only a small increase in conversion from 74 to 76% could be seen with

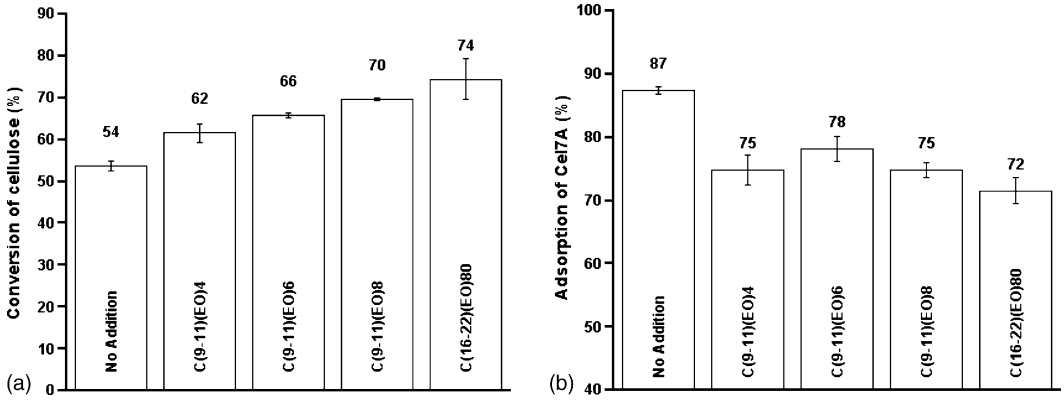

Fig. 1. (a) Conversion of cellulose with addition of ethylene oxide containing surfactants after 16 h hydrolysis of steam-pretreated spruce (SPS). The surfactants had the general formulas $C_{9–11}(EO)_{4, 6, 8}$ and $C_{16–22}(EO)_{80}$ (Table 1). SPS (50 g l^{-1}) was hydrolyzed at pH 4.8 at 40 °C using a cellulase activity of 0.69 FPU ml^{-1} (Celluclast 1.5 l) with β-glucosidase activity (Novozym 188) added at 1.40 IU ml^{-1}. Surfactant concentration was 2.5 g l^{-1}. (b) Adsorption of *T. reesei* Cel7A (CBH I). Adsorption was measured by adding tritium labeled Cel7A to the enzyme mixture to a concentration of 0.01 μM. Labeled Cel7A in solution was quantified radiometrically. All experiments were performed in triplicate and mean values and standard deviations are presented.

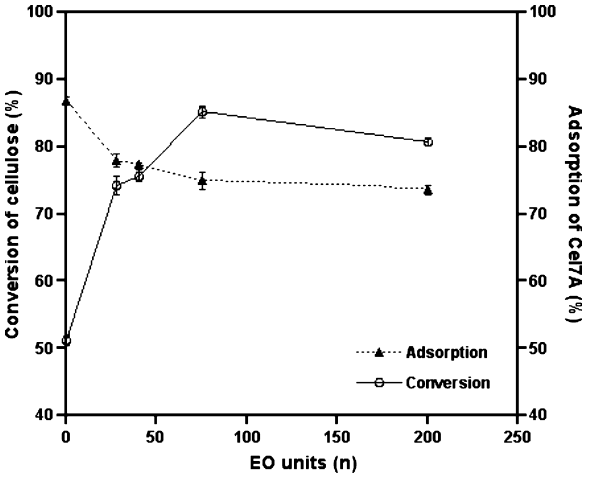

Fig. 2. Adsorption of Cel7A and conversion of cellulose with addition of ethoxylated ricin oil surfactants after 16 h hydrolysis of SPS. The conversion is shown as function of number of EO units in the surfactant hydrophilic part. SPS (50 g l^{-1}) was hydrolyzed at pH 4.8 at 40 °C using a cellulase activity of 0.69 FPU ml^{-1} (Celluclast 1.5 l) with β-glucosidase activity (Novozym 188) added at 1.40 IU ml^{-1}. Surfactant concentration was 2.5 g l^{-1}. Tritium labeled Cel7A was added to the enzyme mixture to a concentration of 0.01 μM. All experiments were performed in triplicate and mean values and standard deviations are presented.

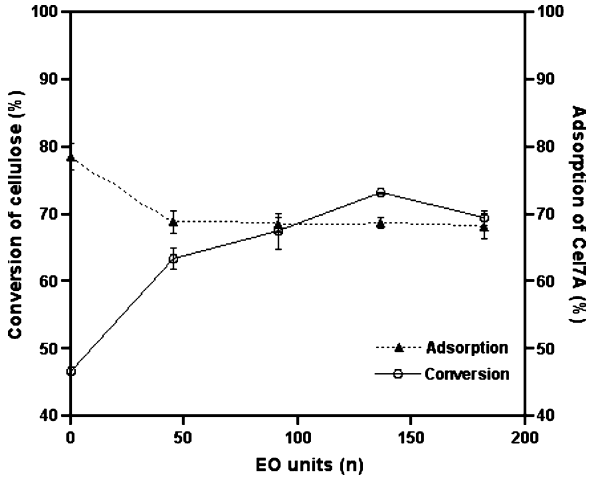

Fig. 3. Adsorption of Cel7A and conversion of cellulose with addition of poly(ethylene glycol) with average molecular masses of 2000 (45 EO), 4000 (91 EO), 6000 (136 EO) and 8000 (182 EO). Conversion is shown as function of number of EO units. SPS (50 g l^{-1}) was hydrolyzed for 16 h at pH 4.8 at 40 °C using a cellulase activity of 0.69 FPU ml^{-1} (Celluclast 1.5 l) with β-glucosidase activity (Novozym 188) added at 1.40 IU ml^{-1}. PEG concentration was 2.5 g l^{-1}. Tritium labeled Cel7A was added to the enzyme mixture to a concentration of 0.05 μM. All experiments were performed in triplicate and mean values and standard deviations are presented.

increase from 28 to 40 EO units. A significant increase in conversion from 76 to 85% ($P \leq 0.05$) was observed when increasing the number of EO units from 40 to 75. Addition of ethoxylated ricin oil with 200 EO units did not further increase conversion but resulted in a conversion of 81%. Also with these surfactants the enzyme adsorption (Cel7A) decreased with the length of the EO chain.

3.3. Addition of poly(ethylene glycol)

From the screening of different non-ionic ethylene oxide containing surfactants the effect seemed to depend on the length of the hydrophilic ethylene oxide part. Therefore, experiments were carried out to study the effect of pure poly(ethylene oxide) polymers, i.e. PEG, on the hydrolysis of lignocellulose and adsorption of enzyme (Fig. 3). Four PEGs with average molecular masses of 2000, 4000, 6000 and 8000 g mol^{-1} were added to hydrolysis mixture. The ethylene oxide content of the PEG polymers corresponded to average values of 45, 91, 136 and 182 EO units, respectively. A 16 h hydrolysis with no addition of PEG gave a conversion of 47% whereas all additions of PEG resulted in an increased conversion ranging from 63 to 73%. Conversion increased with increased molecular mass from 63% for PEG 2000 and up to 73% for PEG 6000 whereas PEG 8000 gave a slightly lower conversion of 69.5%. Adsorption of Cel7A was very similar for all four PEGs added and decreased from 78.5% with no addition down to 68–69% with addition of PEG. For further studies PEG 4000 was selected since it is one of the most largely produced poly(ethylene glycol) polymers and thus is a likely candidate to be used in a large-scale process. It was also suitable for basic studies due to the availability of ^{14}C labeled PEG 4000.

3.4. Effect of concentration of PEG 4000

The effect on conversion of SPS and Cel7A adsorption was investigated with different concentrations of PEG 4000 (Fig. 4). Addition of 0.5 g l^{-1} of PEG 4000 only increased the conversion slightly whereas additions of 1 and 2.5 g l^{-1} increased conversion up to 71 and 79%, respectively. The highest conversion of 87% was obtained with 5 g l^{-1} of PEG 4000. Adsorption of Cel7A with additions of 2.5 and 5 g l^{-1} of PEG 4000 was lowered to 72% from 82% without PEG addition.

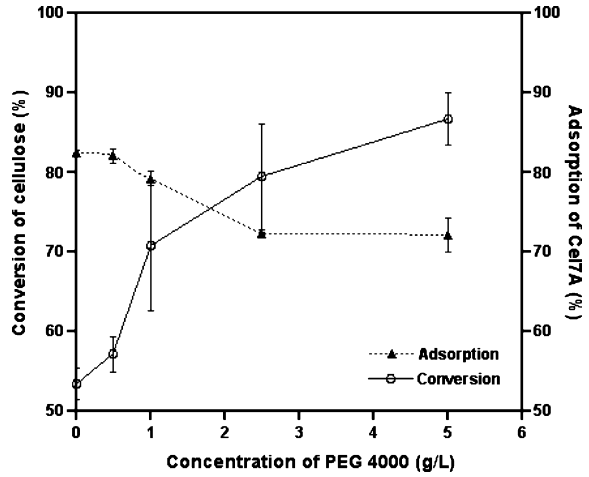

Fig. 4. Adsorption of Cel7A and conversion of cellulose after 16 h hydrolysis as function of PEG concentration. SPS (50 g l^{-1}) was hydrolyzed at pH 4.8 at 40 °C using a cellulase activity of 0.69 FPU ml^{-1} (Celluclast 1.5 l) with β-glucosidase activity (Novozym 188) added at 1.40 IU ml^{-1}. Tritium labeled Cel7A was added to the enzyme mixture to a concentration of 0.1 μM. All experiments were performed in triplicate and mean values and standard deviations are presented.

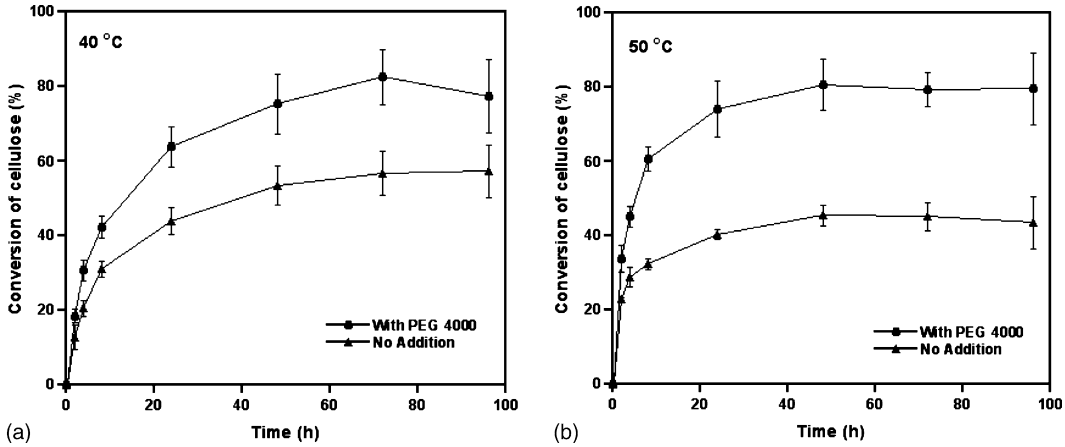

Fig. 5. (a) Time course of SPS hydrolysis with and without addition of PEG 4000. SPS (50 g l^{-1}) was hydrolyzed at pH 4.8 at 40 °C using a cellulase activity of 0.69 FPU ml^{-1} (Celluclast 1.5 l) with β-glucosidase activity (Novozym 188) added at 1.40 IU ml^{-1}. PEG concentration was 2.5 g l^{-1}. (b) Time course of SPS hydrolysis performed at 50 °C, otherwise carried out as presented in (a). All experiments were performed in triplicate and mean values and standard deviations are presented.

3.5. Hydrolysis at higher temperature with addition of PEG 4000

Hydrolysis of SPS with addition of 2.5 g l^{-1} PEG 4000 was investigated at 40 and 50 °C for 96 h (Fig. 5a and b). At 40 °C conversion after 24 h hydrolysis was 44% without addition while 64% conversion was reached with addition of PEG 4000 and the highest conversion of 82.5% was seen after 72 h (Fig. 5a). At the temperature of 50 °C the initial rate of conversion with addition of PEG 4000 was faster compared to 40 °C. Most important, at 50 °C the high conversion rate was maintained with PEG 4000 up to 24 h, whereas without PEG addition the rate started to level off already after 6–8 h. When hydrolysis was performed without addition of PEG 4000 at 50 °C lower conversion was obtained than corresponding experiment at 40 °C. However, with PEG addition the maximum conversion at 50 °C (81%) was reached after only 48 h compared to 72 h (57%) at 40 °C without PEG addition. Thus both high yield and short process time was obtained.

3.6. Hydrolysis of SPS and delignified SPS

To investigate the role of lignin in the effect of PEG during enzymatic hydrolysis of lignocellulosic substrates, experiments were performed on SPS and delignified SPS. The results (Fig. 6a and b) showed that increased conversion due to PEG addition could be correlated to presence of lignin in the substrate. The conversion of SPS to glucose increased from 42 to 78% with addition of PEG 4000 in a 16 h hydrolysis and a significant decrease in adsorption of Cel7A from 81 to 59% was observed ($P \leq 0.05$). In contrast, hydrolysis of delignified SPS was not improved by addition of PEG 4000 and no significant change in enzyme adsorption was observed in this case ($P \geq 0.05$).

3.7. Time course of PEG adsorption to lignocellulose

To investigate the time course of the interaction between PEG 4000 and SPS, 5 g l^{-1} of PEG 4000 with a small amount ^{14}C labeled PEG 4000 was incubated with 50 g l^{-1} of SPS.

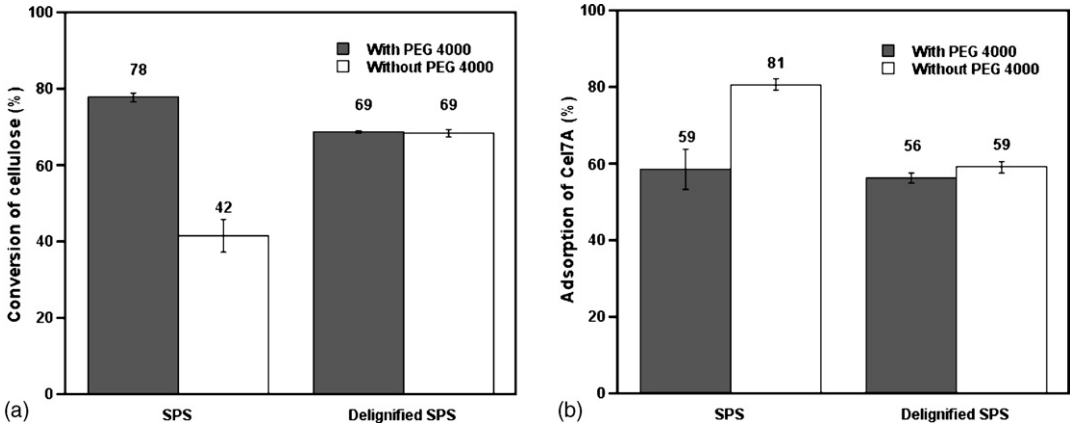

Fig. 6. (a) Hydrolysis of SPS and delignified SPS with and without addition of PEG 4000. Substrates were added to give the same cellulose concentration of 25 g l^{-1}. Hydrolysis was performed at pH 4.8 and 40 °C using a cellulase activity of 0.69 FPU ml^{-1} (Celluclast 1.5 l) with β-glucosidase activity (Novozym 188) added at 1.40 IU ml^{-1}. PEG concentration was 2.5 g l^{-1}. (b) Adsorption of Cel7A in hydrolysis experiment presented in (b). Tritium labeled Cel7A was added to the enzyme mixture to a concentration of 0.1 μM. All experiments were performed in triplicate and mean values and standard deviations are presented.

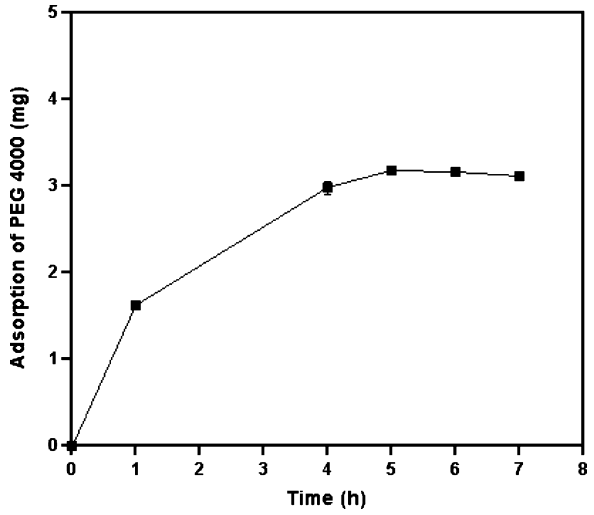

Fig. 7. Time course of PEG adsorption on lignocellulose. SPS (50 g l^{-1}) was incubated with PEG 4000 at a concentration of 5 g l^{-1} at pH 4.8 and 40 °C. ^{14}C radioactively labeled PEG 4000 was added to a concentration of 0.25 μM to measure PEG 4000 adsorption. Labeled PEG 4000 in solution was quantified radiometrically. All experiments were performed in triplicate and mean values and standard deviations are presented.

The results show an increase of PEG adsorption during 5 h where after a maximum level of adsorbed PEG was reached (Fig. 7).

The adsorption behaviour of PEG 4000 was followed during enzymatic hydrolysis of SPS using radioactive labeled PEG (Fig. 8). Adsorption of PEG increased for the first 5–6 h up to a level of 1.5 mg PEG adsorbed. From 6 to 72 h the PEG adsorption increased very slowly up to 1.7 mg PEG adsorbed, in agreement with results without enzymes added (Fig. 7).

Due to the relative slow kinetics of PEG adsorption (Fig. 7) it was interesting to examine if positive effects on conversion could be obtained by pre-incubation of SPS with PEG 4000. Enzymatic hydrolysis was performed for 16 h at 40 °C with a 5 h pre-incubation of PEG 4000 with SPS and compared to experiments with no addition and where PEG 4000 was added at the start of hydrolysis. Results (not shown) clearly showed that pre-incubation with PEG could not increase conversion. With no addition of PEG 4000 conversion of SPS was 42% and the pre-incubated and non-incubated experiment gave conversions of 63 and 64%, respectively.

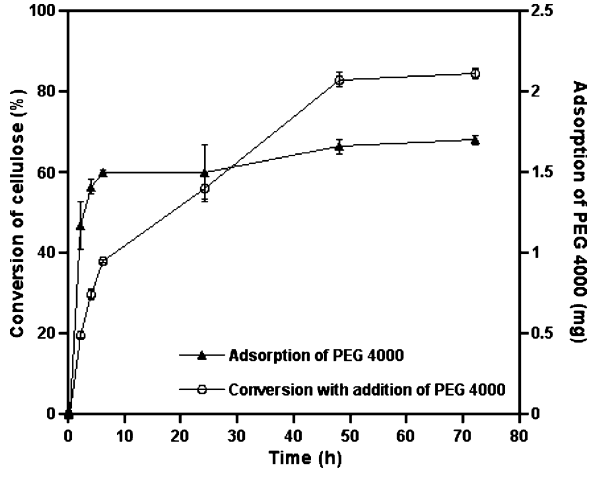

Fig. 8. Time course of PEG adsorption during cellulose conversion. SPS (50 g l^{-1}) was hydrolyzed at pH 4.8 and 40 °C using a cellulase activity of 0.69 FPU ml^{-1} (Celluclast 1.5 l) with β-glucosidase activity (Novozym 188) added at 1.40 IU ml^{-1}. PEG concentration was 2.5 g l^{-1}. ^{14}C radioactively labeled PEG 4000 was added to a concentration of 0.25 μM to measure PEG 4000 adsorption. Labeled PEG 4000 in solution was quantified radiometrically. All experiments were performed in triplicate and mean values and standard deviations are presented.

3.8. Adsorption isotherm for binding of PEG 4000 on SPS

An adsorption isotherm was determined for the interaction between PEG 4000 and SPS by use of ^{14}C labeled PEG 4000 (Fig. 9). The isotherm data were compared with model isotherms using the one and two-site Langmuir models [20] (Eqs. (1) and (2)). The models describe how the adsorbed amount of ligand (poly(ethylene glycol) monomer) depends on the concentration of ligand in solution. In the notations used below [B] stands for the amount of bound PEG (mmol monomer g^{-1} substrate) and [F] is the concentration of free PEG in solution (mmol monomer l^{-1}). The model assumes adsorption of a monolayer where no interactions occur between the adsorbing ligands. The number of binding sites per gram of substrate is denoted n and has the unit of (mmol g^{-1}). K_a (l $mmol^{-1}$) is the association constant of the PEG-lignin complex.

$$[B] = \frac{(n_1 K_{a1}[F])}{(1 + (K_{a1}[F]))} \quad (1)$$

$$[B] = \frac{(n_1 K_{a1}[F])}{(1 + (K_{a1}[F]))} + \frac{(n_2 K_{a2}[F])}{(1 + (K_{a2}[F]))} \quad (2)$$

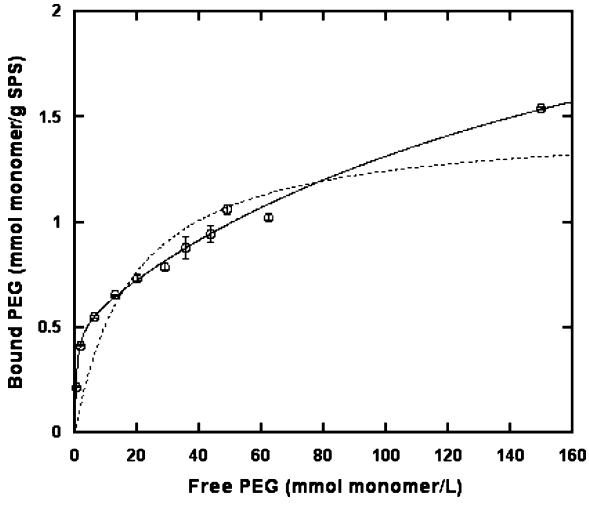

Fig. 9. Adsorption isotherm for PEG 4000 on SPS. For each PEG concentration, SPS (50 g l^{-1}) was incubated over night with PEG 4000 at pH 4.8 and 40 °C. Adsorption of PEG 4000 was quantified radiometrically by addition of ^{14}C labeled PEG 4000. Adsorption is expressed as concentration of monomer (EO units) bound per gram of substrate as a function of concentration of unbound (free) monomers. Fitted isotherms using one site Langmuir model (- - - -) and two-site Langmuir model (—). All experiments were performed in triplicate and mean values and standard deviations are presented.

The relative association constant is denoted Kr ($l\,g^{-1}$) and is defined as ($x = 1$ and 2):

$$Kr_x = n_x K_{ax} \quad (3)$$

A one site Langmuir isotherm did not fit well with the adsorption data (R^2 value of 0.812) (Fig. 9). The two-site Langmuir isotherm (Fig. 9) gave a good fit with a R^2 of 0.992. The relative association constants (Eq. (3)) for the interactions using the two-site Langmuir model were $Kr_1 = 0.012$ and $Kr_2 = 0.945$ for the two different classes of adsorption sites.

4. Discussion

Surface active additives have been presented for enhancement of enzymatic hydrolysis of lignocellulose and several different suggestions of mechanisms behind this effect have been put forward. Kaar and Holtzapple suggested that Tween as additive in enzymatic hydrolysis of corn stover can act as an enzyme stabilizer, lignocellulose disrupter and as an enzyme effector [11]. Sewalt et al. showed the inhibitory effect of pine lignin in enzymatic hydrolysis of filter paper and the reversal of inhibition by addition of various proteins and polymers [9]. Tween has also been shown to decrease process time and increase ethanol yield in simultaneous saccharification and fermentation [21]. Most of the additives shown to have effect on enzymatic conversion of lignocellulose are composed of ethylene oxide containing surfactants and polymers [9,11–13,15]. The presence of lignin has been shown to play a major role for the degradability of lignocelluloses [2,8,10] and the effect of additives has been correlated to the presence of lignin in the substrate [9,13]. In a previous study a mechanism for the effect of surfactants has been suggested [13]. It was here proposed that the hydrophobic part of the surfactant binds to lignin and the hydrophilic part of the surfactant acts as a steric hindrance blocking the enzymes from binding unproductively to lignin. The increased conversion of lignocellulose was thus caused by more enzymes being available for cellulose hydrolysis.

4.1. Surfactants with increased EO chain length

When investigating suitable surfactants to be further evaluated in large scale ethanol production an interesting pattern was found. An increase in conversion with longer ethylene oxide chains was observed followed by a decreased enzyme adsorption. The three surfactants with 4, 6 and 8 EO units had the same hydrophobic part. Also with ricin oil based surfactants similar results were obtained. Earlier results with surfactants have been interpreted based on the role of the hydrophobic alkyl chain adsorbing to lignin [13]. However, increased EO chain length leads to *decreased* surfactant hydrophobicity, as can be seen in the surfactant CMC, e.g. for $C_{10}EO_6$ CMC is 0.9 mM and for $C_{10}EO_9$ 1.3 mM [22]. Yet with an increase of the hydrophilic part of the surfactants the effect on conversion was larger. Thus the properties of the hydrophilic part are more important than previously discussed.

4.2. Addition of poly(ethylene glycol)

The results with ethylene oxide containing surfactants raised the interest to perform experiments where the hydrophobic part of the surfactant was absent, i.e. addition of poly(ethylene oxide) (PEG) polymers. Interestingly the effect of PEG polymers on the conversion was as strong as with non-ionic surfactants and followed the same pattern with increased conversion with increasing length of ethylene oxide chain. If one compares the addition of PEG 4000 (91 EO) and $C_{16–22}80$ EO an increase in the same range was obtained with a slightly higher value for PEG 4000 lacking the hydrophobic alkyl chain. Thus the surfactant alkyl chain is not solely responsible for the positive effect on conversion, but interactions between ethylene oxide and the substrate are dominating. Interaction between PEG and lignin has earlier been shown mainly in connection with pulping processes. Lindström and Glad-Nordmark successfully managed to flocculate unbleached kraft pulps, soda pulps and an unbleached sulphite pulp by using poly(ethylene oxide) [23]. Methylation or acetylation of the pulps removed the possibility for poly(ethylene oxide) to hydrogen bond with phenolic protons on the lignin [23]. Flocculation by PEO in papermaking has been studied by van de Ven [24]. Kadla and Kubo studied composite materials containing a blend of poly(ethylene oxide) and Kraft Lignin, and FT-IR analyses revealed hydrogen bonding interactions between the aromatic hydroxyl proton and the ether oxygen in poly(ethylene oxide) [25].

Explanations for the increasing conversion with the length of EO chain could be that: (i) when the length of the EO chain increases, the polymer binding to lignin is enhanced; (ii) when PEG adsorbs to the substrate surface, EO chains extending out from the lignin surface will occupy a volume excluding enzymes from adsorbing. Excluded volume interaction is a known mechanism for surface bound PEG polymers to reduce protein adsorption on surfaces [26]. This will force enzymes out into the solution making more enzymes available for cellulose degradation. Excluded volume interactions are increased with longer polymer chains [26]. Adsorption of enzyme on the substrate was also significantly reduced when using PEG, similar to the effect of surfactants [13].

Adsorption of PEG 4000 on SPS was best fitted to a two-site Langmuir isotherm. The relative association constants suggest that there is one stronger and one weaker interaction. The structure model of softwood lignin is based on phenylpropane units covalently linked in a network [27]. The amount of aromatic and aliphatic hydroxyls per C_9-unit in spruce milled wood lignin has been determined by Månsson to be 0.93 and 0.33 units, respectively [28]. The proposed model structure together with the analysis of hydroxyl content shows that the spruce lignin structure present good opportunities for both hydrophobic and hydrogen bonding interaction for PEG. The hydrophobic parts of lignin such as phenyl, CH_2 and CH_3 groups will thus interact with the CH_2 groups in PEG. Hydrogen bonding interactions have been shown between phenolic hydroxyls and the ether oxygens in PEG [25]. This suggests that the two PEG-lignin interaction mechanisms are hydrogen bonding and hydrophobic interaction, where both types of interactions are complementary

and will reinforce each other. Comparison can be made with the interactions utilized in the formation of double-stranded polynucleotides (DNA, RNA) due to hydrogen bonding between bases reinforced by hydrophobic stacking interactions. The hydrophobic interaction of enzymes with lignin is also discussed by Palonen et al. [29] where the adsorption of Cel7A (CBH I) and Cel5A (EG II) was studied on both cellulosic and isolated lignin materials.

4.3. Concentration of PEG 4000

The effect of PEG concentration on cellulose conversion and adsorption of Cel7A follows a similar pattern as for surfactants [13]. The similarity in profiles when using PEG and surfactants suggests that a similar mechanism is at work in both cases. The results indicate that a PEG concentration of 2.5 g l^{-1} is sufficient to block most of the lignin surface of SPS as was also obtained with non-ionic surfactants [13]. Addition of 2.5 g l^{-1} PEG corresponds to 0.05 g PEG g^{-1} dry biomass. Kaar and Holtzapple obtained similar results with addition of Tween 80 to hydrolysis of corn stover where the largest increase in conversion was seen with surfactant additions between 0.05 and 0.1 g g^{-1} dry biomass [11]. The feasibility of PEG addition at a concentration of 2.5 g l^{-1} in a subsequent fermentation or in a simultaneous saccharification and fermentation should not result in any negative effects. Larsson et al. showed that both high and low molecular weight PEG at a concentration of 5% (50 g l^{-1}) increased ethanol production in glucose fermentations by *Saccaromyces cerevisiae* at expense of cell mass production [30]. In the same study addition of PEG 8000 (5%) did not influence the cell growth.

4.4. Hydrolysis at higher temperature with addition of PEG 4000

Enzymatic hydrolysis of steam-pretreated softwood without addition of PEG has been optimized to be 40 °C [31]. With PEG addition enzymatic hydrolysis could be performed effectively at a temperature of 50 °C. However, without PEG the conversion was lower at 50 °C compared with 40 °C, probably due to denaturation at the higher temperature. This is in accordance with results by Kaar and Holtzapple showing that addition of EO-containing surfactants (Tween) allow hydrolysis at 50 °C [11].

The possibility to use enzymes at 50 °C can partly be explained by release of enzymes by PEG from lignin surfaces which increase effective enzymes for cellulose hydrolysis, thus leading to increased conversion before enzymes are denatured. The protective effect by PEG and surfactants of the enzymes should also be considered. Kim et al. discuss the denaturation of cellulases at 50 °C in absence of substrate to be caused by enzyme in contact with the liquid–air interface [32]. Surface active compounds compete with enzymes for this surface and may therefore protect them from denaturation [32]. The same mechanism could be relevant for enzymes at the solid–liquid interface in enzymatic conversion of lignocellulose substrate. The higher temperature increases the unfolding of enzymes and due to hydrophobic interactions on the lignin surface, denaturation occurs. The exclusion of enzymes from the lignin surface by PEG leads to that more enzymes are free to adsorb to the natural substrate cellulose, thus enzymes are transferred to a more stable environment.

4.5. PEG—lignin interaction

The hydrolysis of SPS and delignified SPS was carried out to show the role of lignin in enzymatic conversion of lignocellulose and the mechanism of the PEG effect, i.e. interactions with lignin. Increase in conversion could be seen only in the case of the lignin containing substrate, which also was found for surfactants [13]. Earlier results in the same direction are by Sewalt et al., who showed the negative effect of lignin by adding lignin to enzymatic hydrolysis of filter paper [9]. Ooshima et al. showed that a significant amount of cellulase was found to adsorb on the lignocious residue during hydrolysis of pre-treated hardwood [33].

It takes 5–6 h for PEG to reach the binding equilibrium with SPS but already after 1 h more than 50% of the maximum adsorption had been reached. During hydrolysis the effect from PEG addition was seen almost immediately (Fig. 5a). Thus, even though it takes a relatively long time for PEG to reach equilibrium adsorption, the blocking of lignin surfaces by PEG at the beginning of the hydrolysis is enough to achieve higher conversion. This was also shown by the lack of further improvement by pre-incubation of PEG with the substrate for 5 h before initiation of hydrolysis.

In a hydrolysis mixture where both enzymes and PEG are competing for adsorption on lignin surfaces it also takes 5–6 h for PEG to reach maximum adsorption on the substrate. The small increase in PEG adsorption seen after this time is most likely due to newly exposed lignin surfaces from the degradation of cellulose.

5. Conclusions

Addition of non-ionic surfactants and polymers containing poly(ethylene oxide) can effectively increase enzymatic hydrolysis of lignocellulose. The length of ethylene oxide chains affects the conversion of cellulose and the adsorption of the dominating *T. reesei* cellulase Cel7A (CBHI). For the PEG lengths investigated an increase could be seen up to PEG 6000, thereafter no further increase was observed. Addition of PEG also facilitates the use of a higher process temperature (50 °C), resulting in shorter process time. Cellulose conversion of 82% was obtained already after 48 h at 50 °C. The reduced process time should be economically advantageous in a separate hydrolysis and fermentation process configuration. PEG was shown only to have an effect on hydrolysis when lignin was present in the substrate. The adsorption of PEG on lignocellulose is proposed to be due to hydrophobic and hydrogen bonding interactions between PEG and the lignin part in the lignocellulose. Based on the similar effect on conversion when comparing $C_{16–22}$EO80 and PEG 4000 (91 EO) it is evident that poly(ethylene oxide) within these additives adsorb to lignin. The excluded volume interactions by

adsorbed PEG polymers on the lignocellulose surface will hinder enzymes from binding unproductively on the lignin surface. A higher concentration of enzymes is therefore available for cellulose degradation.

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