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Dissecting the effect of chemical additives on the enzymatic hydrolysis of pretreated wheat straw

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HIGHLIGHTS

- Enzymatic hydrolysis of pretreated wheat straw in the presence of chemical additives.
- Final substrate conversion enhanced by urea.
- Late-stage hydrolysis rate enhanced by cetyl-trimethylammonium bromide.
- Enzyme adsorption mainly affected by PEG8000.
- Synergistic effect of combination of PEG8000 and urea.

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ABSTRACT

Chemical additives were examined for ability to increase the enzymatic hydrolysis of thermo-acidically pretreated wheat straw by *Trichoderma reesei* cellulase at 50 °C. Semi-empirical descriptors derived from the hydrolysis time courses were applied to compare influence of these additives on lignocellulose bioconversion on a kinetic level, presenting a novel view on their mechanism of action. Focus was on rate retardation during hydrolysis, substrate conversion and enzyme adsorption. PEG 8000 enabled a reduction of enzyme loading by 50% while retaining the same conversion of 67% after 24 h. For the first time, a beneficial effect of urea is reported, increasing the final substrate conversion after 48 h by 16%. The cationic surfactant cetyl-trimethylammonium bromide (CTAB) enhanced the hydrolysis rate at extended reaction time (r_{lim}) by 34% and reduced reaction time by 28%. A combination of PEG 8000 and urea increased sugar release more than additives used individually.

1. Introduction

Lignocellulosic biomass is a potential feedstock for production of second-generation biofuels and other (bio)chemicals. However, several technical and economic factors still prevent the large-scale utilization of lignocellulose as a source for fermentable sugars, most notably a rapid decrease in hydrolysis rate and a need for high enzyme loadings (Himmel et al., 2007). Enzyme-associated factors such as product inhibition, enzyme denaturation, enzyme clogging or jamming as well as substrate-associated factors such as decreasing substrate reactivity and accessibility, multiphasic composition of cellulose, changes in degree of polymerization or crystallinity have been held responsible for sugar production at low space–time yields (Bansal et al., 2009; Bubner et al., 2012; Eibinger et al., 2014; Zhang and Lynd, 2004; Zhao et al., 2012).

To overcome these challenges and improve the economic viability of enzymatic hydrolysis, different strategies have been applied: Screening of microorganisms for new lignocellulose-degrading enzymes with better performance (Marjamaa et al., 2013), protein engineering (Thongekkaew et al., 2013), improvement of pretreatment technologies to increase cellulose accessibility (Agbor et al., 2011) or recovery and reuse of cellulases to reduce enzyme cost (Rodrigues et al., 2012). One promising approach to increase the enzymatic hydrolysis of cellulose is the supplementation of surfactants (e.g. Tween, Q-86W, Triton, Anhitole 20 BS), polymers (e.g. poly(ethylene glycol) (PEG), ricin oil ethoxylate, alcohol ethoxylate) or non-catalytic proteins (e.g. bovine serum albumin (BSA)) (Börjesson et al., 2007; Eriksson et al., 2002; Kristensen et al., 2007; Ooshima et al., 1986). It has been concluded that non-ionic

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surfactants are most effective in improving cellulose hydrolysis (Ooshima et al., 1986). Supplementation of poly(oxyethylene)$_{20}$ sorbitan monooleate (Tween 80) during cellulase hydrolysis of pretreated lodgepole pine resulted in a 32% increase of cellulose-to-glucose yield and reduced the hydrolysis time by 50%, while enhancing the amount of free enzyme in the hydrolysate (Tu et al., 2009). Ethylene oxide polymers like PEG showed a similar effect, increasing the conversion while enhancing the amount of free enzymes in the liquid phase and reducing process time (Ouyang et al., 2011). The main mechanism of surfactants, polymers and non-catalytic proteins might be a prevention of unspecific enzyme adsorption on lignin (Eriksson et al., 2002). Non-biospecific adsorption of proteins on lignin induces protein unfolding and enzyme inactivation, contributing to the excessive consumption of enzymes (Helle et al., 1993). Surfactants and polymers are believed to form a hydrated layer on the lignin surface, presenting a steric hindrance to unproductive cellulase binding. Thus, more enzymes are available for cellulose hydrolysis (Eriksson et al., 2002). Polymers and surfactants might also disrupt the (ligno)cellulose structure by removing lignin or amorphous cellulose, reinforce biomass swelling and increase cellulose accessibility (Helle et al., 1993; Kaar and Holtzapple, 1998; Li et al., 2012). Besides substrate-related mechanisms, a stabilizing effect on enzyme activity has also been suggested. Surfactants and polymers could protect enzymes from thermal denaturation, impede aggregation of instable cellulose components and help enzymes to desorb from the binding site after completion of saccharification (Helle et al., 1993; Kaar and Holtzapple, 1998).

Previous studies investigating the effect of additives on lignocelluloses hydrolysis focused mainly on wood materials, especially softwood lignocellulose (Börjesson et al., 2007; Seo et al., 2011; Tu et al., 2009). Wheat straw (*Triticum aestivum*) is a highly abundant, low-cost agricultural residue, which is not fully exploited today, therefore an interesting raw material for bioethanol production. Compared to wood biomass, herbaceous lignin has a different monomeric composition, containing guaiacyl, syringyl and *p*-hydroxyphenyl units in significant amounts. Lignins are acylated by *p*-coumaric acid at the γ-position of lignin sidechains, and lignin–carbohydrate complexes contain ferulic acid bridges between lignin and hemicellulose. These differences could possibly influence enzyme–substrate interactions and the effect of surfactants (Buranov and Mazza, 2008; Kristensen et al., 2007). A better understanding of the connections between chemical additives, enzyme adsorption and cellulose saccharification is crucial for making adaptive changes in the hydrolysis process. In a recent publication, we established a set of simple kinetic descriptors facilitating the correlation of enzyme adsorption and cellulose hydrolysis (Monschein et al., 2013). By applying these descriptors to the enzymatic hydrolysis of thermo-acidically pretreated wheat straw, the effects of non-ionic surfactants poly(oxyethylene)$_{20}$ sorbitan monolaurate (Tween 20), Tween 80, octyl-phenol(ethylene glycol)$_{7,5}$ ether (Triton X-100); cationic surfactant cetyltrimethylammonium bromide (CTAB); chaotropic agent urea; and the uncharged polyether polymers PEG 2000 and PEG 8000 were compared on a kinetic level, presenting a novel view on their mechanism of action.

2. Methods

2.1. Substrates and chemicals

Thermo-acidically pretreated wheat straw with a total dry matter content of 20.7% was supplied by CLARIANT (Munich, Germany). Wheat straw was pretreated in a high pressure autoclave at 145 °C for 13 min. A liquor ratio of 6.5:1 was used, and the liquid was supplemented with 0.5% H_2SO_4. The dry matter was composed of 58.1% glycan, 25.6% lignin, 4.9% inorganic substances, 2.1% water-soluble substances and 1.7% acid. Glucose accounted for 86.1% of the total sugar content, with xylose (12.1%), fructose (1%), arabinose (0.5%) and mannose (0.3%) making up the remaining portion. Roti-Quant reagent, Roti-Nanoquant reagent, CTAB, urea and Triton X-100 were from Carl Roth (Karlsruhe, Germany). PEG 2000 and PEG 8000, Tween 20, Tween 80, 4-nitrophenol and 4-nitrophenyl-β-D-glucopyranoside were from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were reagent grade.

2.2. Enzymes

ENZ-SC01 cellulase-mixture, containing the complete enzyme system of *Trichoderma reesei*, was from CLARIANT. We determined cellulase activity as 27 FPU/mL, β-glucosidase activity as 52 U/mL and protein content as 20 g/L.

2.3. Measurement of enzyme activities

Cellulase activities were measured in duplicates using the standardized FPU assay (Ghose, 1987). For measurement of β-glucosidase activity, the nitrophenol assay, calibrated against 4-nitrophenol, was used. The assay was performed in 96-well flat-bottom microtiter plates (GreinerBio-One International AG, Frickenhausen, Germany). Fifty microliters of 4-nitrophenyl-β-D-glucopyranoside were mixed with 50 μL of sample diluted in 50 mM Na-acetate buffer, pH 5.0, and incubated for 10 min at 50 °C and 300 rpm in an Eppendorf Thermomixer comfort (Eppendorf AG, Hamburg, Germany). After 5 min on ice, reactions were stopped by addition of 100 μL of 1 M Na_2CO_3. Absorbances were measured at 405 nm against a blank of substrate incubated with Na-acetate buffer in a FLUOstar Omega plate reader (BMG Labtech GmbH, Ortenberg, Germany).

2.4. Enzymatic hydrolysis with supplementation of various additives

Hydrolysis experiments were performed in duplicates and replicated twice. A suspension of pretreated wheat straw (25 g/L dry matter; 14.53 g/L glycan) was prepared in a total reaction volume of 1.8 mL of 50 mM Na-acetate buffer (pH 5.0). The substrate suspension was supplemented with one of the tested additives: PEG 2000 (0.5–2.5 g/L), PEG 8000 (0.5–2.5 g/L), CTAB (0.125–0.5 g/L), urea (0.5 g/L), Triton X-100 (0.5 g/L), Tween 20 (0.125 g/L) or Tween 80 (0.125 g/L). ENZ-SC01 was added in a concentration of 25 FPU/g glycan. Reaction tubes were incubated at 50 °C and 1000 rpm for 48 h using an Eppendorf Thermomixer comfort. At certain times, samples were taken. After a brief centrifugation (9300*g*, 30 s) 100 μL of the supernatant was retained for protein determination. The remaining sample was heated to 95 °C for 7 min and the cleared supernatant used for sugar analysis. Control reactions were performed without additive supplementation.

For testing the influence of enzyme loading on the effect of PEG 8000, different concentrations of ENZ-SC01 (from 6.2 to 62 FPU/g glycan) were added to the substrate suspension supplemented with 2.5 g/L PEG 8000. Hydrolysis was performed as stated above. Control reactions were performed without additive supplementation.

The combined effect of different additives was investigated by supplementing the substrate suspension with two different additives in three possible combinations: PEG 8000/CTAB, PEG 8000/urea or CTAB/urea. The additives were added in concentrations ranging from 6.3×10^{-2} to 5 g/L. ENZ-SC01 was added in a concentration of 25 FPU/g glycan. Hydrolysis was performed as stated above. Control reactions were performed with and without supplementation of the individual additives.