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Short Communication

The effect of bovine serum albumin on batch and continuous enzymatic cellulose hydrolysis mixed by stirring or shaking

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ABSTRACT

Bovine serum albumin (BSA) was applied as a model non-catalytic protein to enzymatic hydrolysis of Avicel and dilute acid pretreated corn stover at different reaction conditions to improve the understanding of its ability to enhance cellulose hydrolysis. Addition of BSA improved the 72 h hydrolysis yields in shake flasks by up to 26% for both substrates by reducing de-activation of the exoglucanases and by facilitating reductions in particle size and crystallinity during a magnetically stirred pre-incubation step. The enzyme stabilizing effect of BSA addition was most striking for batch hydrolysis in a stirred tank reactor, with glucose yields increasing by 76% after 72 h for Avicel and by 40% after 145 h for corn stover. Application of BSA to continuous hydrolysis for a mean residence time of 24 h gave 33% and 40% higher glucose yields for corn stover and Avicel compared to the controls.

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1. Introduction

The use of additives such as surfactants or non-catalytic proteins such as bovine serum albumin (BSA) has been shown to enhance cellulose hydrolysis by enzymes, potentially lowering costs. While most studies focused on the use of different surfactants, only few reports are available for the use of BSA as an additive. Eriksson et al. (2002) tested the effect of BSA addition at a concentration of 17 g L⁻¹ on hydrolysis of steam-pretreated spruce and compared it with the effect of different surfactants. Both BSA and Tween 20 improved the 24 h hydrolysis yield from approximately 35% to 50%, but combined addition did not lead to further improvement. It was postulated that BSA adsorbed on lignin and thereby prevented unproductive binding of cellulase on these binding sites. This hypothesis is supported by a study about the adsorption behavior of the exoglucanase CBH I and the endoglucanase EG II, where it was found that in the presence of excess BSA, both enzymes bound to a lesser extent to cellulase enriched lignin and the amount of free enzyme in solution increased from 22% to 49% (Palonen et al., 2004). Adding 1% BSA also improved 72 h hydrolysis yields from dilute acid pretreated corn stover,

ammonia fiber expansion pretreated corn stover, and SO₂ steam exploded Douglas fir by approximately 10% by increasing filter paper activity in solution by a factor of 2 and the amount of β-glucosidase activity by a factor of 14 (Yang and Wyman, 2006). One objective of this work was to optimize BSA supported hydrolysis of Avicel and dilute acid pretreated corn stover with respect to additive concentration and investigate the influences of mixing by stirring or shaking and hydrolysis temperature on the BSA effect. A second objective was to determine the effect of BSA addition on performance of continuous hydrolysis based on the idea that continuous processing would benefit more from BSA through making more enzyme available for hydrolysis.

2. Methods

2.1. Substrates

Corn stover was milled through a 2 mm screen and then pretreated for 40 min at 140 °C with 1% H₂SO₄ at a 5% solids loading in a 1 L stirred tank reactor (4520 Series, Parr Instruments, Moline, IL) equipped with a double stacked pitch blade impeller running at 800 rpm. After pretreatment, the slurry was filtered, and the remaining solids were thoroughly washed with water. The composition of the pretreated corn stover was determined according to standard procedures published by the National Renewable Energy Laboratory (Sluiter et al., 2008) and contained 64.1% glucan, 3.4% xylan and 26.3% lignin. Microcrystalline cellulose Avicel PH101 (Sigma Aldrich, St. Louis, MO) was used without modification.

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2.2. Batch hydrolysis experiments

Slurries of Avicel or pretreated corn stover containing 1% cellulose were enzymatically hydrolyzed in 0.05 M citric acid buffer at pH 4.8 supplemented with 10 mg L⁻¹ NaN₃ employing Spezyme CP cellulase (Genencor, Palo Alto, CA, USA) and Novozyme 188 β-glucosidase (Novozyme, Franklinton, NC, USA) at a temperature of 50 °C, unless otherwise noted. The cellulase loading was set to 10 FPU (g glucan)⁻¹ and 15 CBU (g glucan)⁻¹ for Avicel hydrolysis and to 2.5 FPU (g glucan)⁻¹ and 3.75 CBU (g glucan)⁻¹ for corn stover in order to achieve approximately identical yields for both substrates. The enzyme additions correspond to a protein loading of approximately 0.2 and 0.05 g L⁻¹, respectively. The experiments were carried out in triplicates and the average values are presented together with the standard deviation of the mean.

Shaken hydrolysis experiments were performed in 125 mL Erlenmeyer flasks that contained a total reaction mass of 25 g and were placed on a shaking incubator featuring a throw of 25 mm (Multitron 2, Infors-HT, Bottmingen, Switzerland) that rotated at 150 rpm. Prior to cellulase addition to start hydrolysis, the reaction mixtures were supplemented with BSA (Sigma-Aldrich, St. Louis, MO, USA) under 2 different conditions: 1) pre-shaken for 24 h at 50 °C or 2) pre-stirred at room temperature for 24 h with 20 mm magnetic stir bars rotating at 500 rpm. Unless otherwise specified, a BSA concentration of 5 g L⁻¹ was employed, and respective controls without BSA were treated identically.

Stirred hydrolysis experiments were conducted with a total reaction mass of 500 g contained in a 3 L stirred tank bioreactor (Applikon Inc., Foster City, CA, USA) fitted with a 40 mm 8-bladed impeller operating at 500 rpm. BSA was added at a concentration of 5 g L⁻¹ at the same time as the hydrolytic enzymes when the reaction temperature reached 50 °C.

2.3. Continuous hydrolysis

All continuous hydrolysis runs were performed similarly to the batch approach with respect to enzyme loading, solid loading and pH at 50 °C in the 3 L stirred tank bioreactor. Both Avicel and corn stover were magnetically pre-stirred with 5 g L⁻¹ BSA for 24–48 h prior to feeding the substrate to the reactor. The reaction volume was 0.75 L, and the mean residence time was set to 24 h. To supply a homogenous feed of solid substrate and to avoid rapid settling of biomass in the feeding tubes, air bubbles were concomitantly fed into the feeding tube (Weimer et al., 1991). Substrate was fed to the reactor every 6 min, and cellulase from a reservoir cooled to 4 °C was fed continuously with a peristaltic pump.

2.4. Analytical procedures

Sugar concentrations were analyzed using high performance liquid chromatography (HPLC) (Alliance 2695, Waters) equipped

with a refractive index detector (2414, Waters, Milford, MA, USA) and an Aminex HPX-87H column (BioRad, Hercules, CA, USA) with 0.005 M sulfuric acid as the eluent in an isocratic mode at 65 °C. Cellulose crystallinity was determined by X-ray powder diffraction (Siemens Kristalloflex D500) with a step size of 0.5° per minute after freeze drying the samples, with crystallinity calculated based on the Segal method (Thygesen et al., 2005).

3. Results and discussion

3.1. Influence of BSA addition method prior to shaken enzymatic hydrolysis

In this study, we aimed to further elucidate the effect of BSA addition on enzymatic hydrolysis reactions of pure microcrystalline cellulose, i.e., Avicel, and lignin-containing dilute acid pretreated corn stover under a variety of reaction conditions. First, we investigated the effect of a pre-incubation step of biomass with BSA prior to adding enzymes. Slurries of Avicel and dilute acid pretreated corn stover were supplemented with 5 g L⁻¹ BSA and either magnetically stirred or shaken for 24 h prior to start of hydrolysis by addition of cellulase and incubation in a shaking hood. As shown in Table 1, BSA addition in the pre-shaken mode improved the 72 h hydrolysis yield from Avicel by 12.8 ± 1.3%, in contrast to an improvement of 20.7 ± 6.0% by the pre-stirred mode, each compared to the respective control in the same mode. The positive effect of BSA in the pre-shaken mode increased with hydrolysis time as after 24 h there was only a small difference from the control in contrast to the 72 h sampling point. However, the yield improvement due to BSA addition in the pre-stirred mode was observed quickly after a hydrolysis time of only 24 h. For corn stover, the final glucose yield improvement due to the BSA addition was only 4.8 ± 1.8% in the pre-shaken mode, but 18.0 ± 4.3% in the pre-stirred mode. Again, the positive effects of pre-stirring already appeared after a hydrolysis time of only 24 h.

Because yield improvements were only observed after prolonged reaction times for pre-shaken reactions, we hypothesized that one effect of BSA might be the prevention of thermal deactivation of cellulase and tested the effect of BSA at a lower reaction temperature of 38 °C. As shown in Table 1, BSA did not affect Avicel hydrolysis for BSA addition via pre-shaking at a reaction temperature of 38 °C, and the final yield for the control was slightly higher at 38 °C than at 50 °C. However, changing reaction temperature did not alter the effect of BSA on pre-stirred Avicel hydrolysis and on both modes of corn stover hydrolysis, and in these cases all yields were higher at 50 °C.

Taken together, these findings suggest that BSA in the pre-shaken mode reduced de-activation of cellulolytic enzymes during hydrolysis of Avicel. The different results with corn stover suggest that this stabilization is more pronounced or more important for exoglucanases – enzymes that act mainly on crystalline cellulose

Table 1
Influence of temperature and pre-incubation mode on the effect of BSA on the enzymatic hydrolysis of Avicel and pretreated corn stover. Shown are the glucan conversion yields after 24 and 72 h hydrolysis and in brackets the relative increase in yield compared to the respective control reaction due to the addition of 0.5% BSA.

Sample	Pre-incubation mode	T (°C)	Avicel		Corn stover	
			24 h glucan conversion (%) (relative increase (%))	72 h glucan conversion (%) (relative increase (%))	24 h glucan conversion (%) (relative increase (%))	72 h glucan conversion (%) (relative increase (%))
Control	Shaken	50	56.5 ± 1.2	71.7 ± 0.7	46.7 ± 0.8	65.2 ± 0.8
BSA			59.1 ± 0.1 (+4.6 ± 2.2)	80.9 ± 0.5 (+12.8 ± 1.3)	47.1 ± 0.9 (+0.9 ± 2.6)	68.3 ± 0.8 (+4.8 ± 1.8)
Control	Stirred	50	59.6 ± 2.9	78.4 ± 3.8	43.9 ± 0.8	67.6 ± 1.2
BSA			70.3 ± 2.5 (+18.0 ± 7.1)	94.6 ± 1.1 (+20.7 ± 6.0)	52.2 ± 2.0 (+18.9 ± 5.0)	79.8 ± 2.5 (+18.0 ± 4.3)
Control	Shaken	38	52.3 ± 0.4	74.3 ± 0.7	33.3 ± 0.7	53.8 ± 0.7
BSA			51.0 ± 0.1 (-2.5 ± 0.8)	74.4 ± 0.9 (+0.1 ± 1.5)	33.5 ± 0.4 (+0.6 ± 2.4)	57.1 ± 0.4 (+6.1 ± 1.6)
Control	Stirred	38	56.5 ± 3.3	76.5 ± 3.2	29.7 ± 0.6	54.0 ± 1.2
BSA			62.7 ± 1.8 (+11.0 ± 7.2)	85.0 ± 1.0 (+11.1 ± 4.8)	35.8 ± 0.9 (+20.5 ± 3.9)	64.3 ± 1.7 (+19.1 ± 4.1)

(Teeri, 1997). De-activation of cellulases is a known phenomenon: Kim et al. (1982) showed that the air–liquid interface affects cellulase activity far more than shear and that the effect of air–liquid interface decreased with increasing protein concentration. However, surface active agents and BSA (1 g L^{-1}) also reduced the extent of de-activation by decreasing the cellulase concentration at the air–liquid interface. Reese and Mandels (1980) showed that exoglucanase is the least stable component of the cellulase system and that its stability depends on the strain that produced the cellulase. Thus, it is very likely that BSA prevents destabilization of the exoglucanase activity of Spezyme CP and that the inconsistency in literature data on the effect of BSA or surfactants on pure cellulosic substrates where some reported no effect even at long reaction times and $50 \text{ }^\circ\text{C}$ (Yang and Wyman, 2006; Zheng et al., 2008) while others found clear improvements (Borjesson et al., 2007; Kim et al., 1997; Ooshima et al., 1986; Park et al., 1992; Tu et al., 2007) might be explainable by different enzyme sources.

Pre-shaking of dilute acid pretreated corn-stover with BSA gave moderate improvements at both hydrolysis temperatures and already after reaction times of 24 h. Thus, the underlying mechanism likely differs from the stabilization observed for Avicel hydrolysis. The improvement is comparable to literature results and can be explained by prevention of unproductive adsorption of cellulase onto lignin by blocking the binding sites with BSA (Kristensen et al., 2007; Yang and Wyman, 2006; Zheng et al., 2008). As pretreated corn stover is less crystalline than Avicel (see below), the stabilization of exoglucanases seems not to be relevant for the observed hydrolysis yields.

To elucidate the positive effect of pre-stirring the substrates with BSA, the crystallinity index of pre-incubated but undigested samples was measured by X-ray diffraction to determine whether a change in crystallinity could contribute to differences in performance with BSA addition. As shown in Table 2, the crystallinity index of pre-stirred Avicel samples was slightly lower than for samples from pre-shaking, irrespective of whether BSA was added or not. In contrast, pre-stirring of corn stover without BSA reduced crystallinity only slightly to a value of 0.23 compared to the pre-shaken control with a crystallinity index of 0.28, while pre-stirring with BSA lowered the crystallinity index considerably to a value of 0.14 compared to its pre-shaken control (0.27). Because a significant change in particle size could also influence enzymatic hydrolysis, the effect of BSA addition mode on particle size was determined qualitatively by visual inspection under a light microscope. Although no differences were obvious between Avicel samples, pre-stirring of corn stover with BSA resulted in a homogenous suspension of fine particles, with no large particles visible. On the other hand, pre-stirring without adding BSA only changed the appearance of the corn stover particles slightly (data not shown). Thus, the obvious physical changes for corn stover could explain the higher digestibility of pre-stirred material, but it was not clear why BSA facilitated breakdown of the particles.

3.2. Optimization of BSA concentration

Next, we investigated the effect of the BSA concentration on hydrolysis yields in both addition modes in order to optimize the

Table 2
Crystallinity index of Avicel and corn stover after shaken or stirred pre-incubation with and without BSA.

	Pre-shaken	Pre-stirred
Avicel control	0.53	0.48
Avicel with BSA	0.56	0.50
Corn stover control	0.28	0.23
Corn stover with BSA	0.27	0.14

process. For the pre-shaken experiments, BSA concentrations of 0, 0.05, 0.1, 0.5 and 1 g L^{-1} were applied. The 72 h hydrolysis yields increased with increasing BSA concentration up to 0.5 g L^{-1} and then leveled off at a $21 \pm 8\%$ and $11 \pm 3\%$ improvement for Avicel and corn stover, respectively. Application of higher BSA concentrations of 0, 0.1, 0.5, 5 and 20 g L^{-1} for the pre-stirred mode showed that Avicel yields leveled off at maximum improvement of $19 \pm 6\%$ at a BSA concentration of 0.5 g L^{-1} . However, for corn stover, sugar yields increased over the entire range of BSA concentrations applied and reached a $26 \pm 5\%$ improvement at the highest BSA concentration tested.

3.3. Stirred hydrolysis

We also tested the effect of BSA when the hydrolysis reactions were performed in a 3 L stirred tank reactor. Here, BSA was even more effective in that its addition increased glucose yields after 96 h from Avicel from 44.9% to 79.2%, corresponding to an improvement of 76%. The effect was somewhat smaller for corn stover, with the yield increasing from 52.6% to 73.6% corresponding to an increase of 40% after 145 h. The considerably lower yields for the Avicel and corn stover hydrolysis in the stirred tank reactor compared to those with shake flasks are likely due to the shear sensitivity of cellulase reported in the literature: the degree of cellulase de-activation in solution without substrate present was shown to increase with increasing stirrer speed, with a 3% loss in activity at 500 rpm and 28% loss at 2000 rpm, both after 5 h (Ganesh et al., 2000). A more detailed investigation of cellulase shear sensitivity revealed that exoglucanase was most prone to de-activation and could account for the overall loss in cellulase activity measured in substrate free solution (Gunjekar et al., 2001). Based on the experiments reported here, it is not clear whether BSA prevents de-activation at the air–liquid interface or protects enzymes against high shear forces.

3.4. Continuous hydrolysis

The second part of the project focused on determining how beneficial BSA addition could be in continuous hydrolysis. As shown in

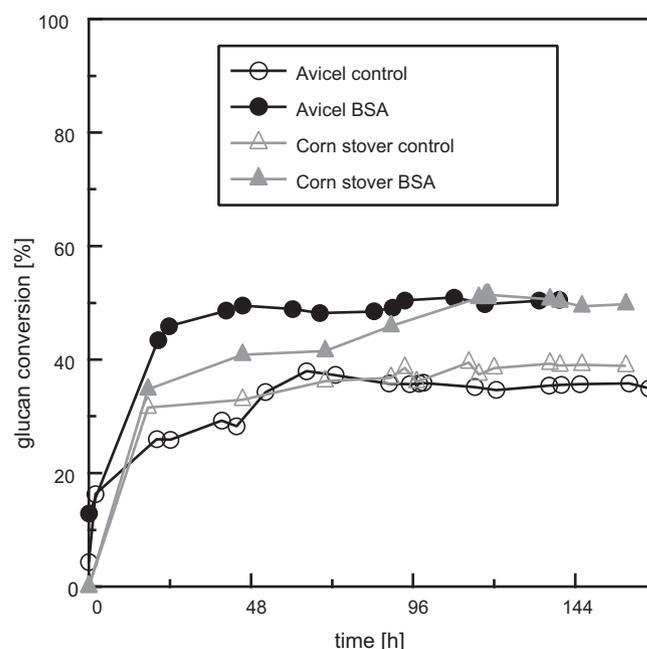


Fig. 1. Glucan conversions over time for continuous hydrolysis of Avicel and corn stover at mean residence times of 24 h for both. The enzyme loading was set to $10 \text{ FPU (g glucan)}^{-1}$ for Avicel hydrolysis and $2.5 \text{ FPU (g glucan)}^{-1}$ for corn stover hydrolysis.

Fig. 1, continuous hydrolysis of Avicel resulted in a glucan conversion of $35.9 \pm 0.6\%$ for the control experiment, and BSA addition increased glucan conversion to $50.2 \pm 0.6\%$, an improvement of $40 \pm 3\%$. The corresponding yields for corn stover were $38.1 \pm 1.2\%$ and $50.8 \pm 0.7\%$, respectively, an improvement of $33 \pm 5\%$ due to BSA addition. The beneficial effects of pre-stirring of the substrate with BSA in the feed tank could explain part of the improvement, as well as the circumvention of enzyme de-activation by shear stress in the stirred tank reactor. In one continuous control experiment, in which Avicel had been pre-stirred with BSA followed by thorough washing with BSA free fresh buffer prior to feeding it to the reactor, a yield improvement of only 12% was measured (data not shown), indicating that the stabilizing effect of BSA contributed a larger share to the overall improvement. On the other hand, in a shake flask batch experiment with a corn stover sample from the feed tank, an improvement of 23% was observed in the presence of BSA; thus a possible stabilizing effect seems to be less important in the case of continuous corn stover hydrolysis which is in agreement with the presented small scale batch experiments.

4. Conclusions

In this work, addition of BSA gave up to 76% higher glucose yields for enzymatic hydrolysis of cellulose. In addition to preventing unproductive binding of cellulase onto lignin as previously described, BSA also appeared to reduce exoglucanase de-activation, thereby improving hydrolysis of microcrystalline cellulose, in particular. Stirring of pretreated corn stover with BSA prior to reaction facilitated decrystallization and size reduction of biomass particles, resulting in a highly digestive substrate.

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