**The role of product inhibition as a yield-determining factor in enzymatic high- solid hydrolysis of pretreated cornstover**

Olsen, SN, Borch, K, Cruys-Bagger N, Westh, P\*

\*Corresponding author:

1Roskilde University

Universitetsvej 1

PO BOX 260

4000 Roskilde Denmark

2Novozymes A/S

Krogshøjvej 36

2880 Bagsværd, Denmark

**Abstract**

Industrially, enzymatic hydrolysis of lignocellulose at high-solid content is preferable over low solids due to a reduction in processing costs. Unfortunately, the economic benefits are counteracted by a linear decrease in yield with solid content, referred to as the “solid effect” in the literature. In the current study we investigate the contribution of product inhibition to the solid effect (**7**-33 % solids). Product inhibition was measured directly by adding glucose to high-solid hydrolysis samples and indirectly through variation of water content and beta-glucosidase concentration. The results suggest that the solid effect is mainly controlled by product inhibition under the given experimental conditions (washed pretreated cornstover as substrate). Cellobiose was found to be approximately 15 times more inhibitory than glucose on a molar scale. However, considering that glucose concentrations are at least 100 times higher than cellobiose concentrations under industrial conditions, glucose inihibition of cellulases is suggested to be a main cause of the solid effect.

**Introduction**

Enzymatic saccharification is a key process in upcoming industries producing liquid fuels from cellulose-rich biomass (*1;2*). This is due to the great abundance of waste crops as wheat straw or corn stover which can be hydrolyzed to fermentable sugars using effective economically feasible enzymes solutions(*1;3;4*).Currently, however, the commercial relevance of bio-ethanol is limited by costs associated with the process step, which includes up to 4 days of enzymatic hydrolysis at 50 oC(*4;5*). Thus, at present, much attention is directed towards optimizing the enzyme complex (by wild type screening, rational design or directed evolution(*4*)) or modulating the reaction conditions (temperature, water content, physical property of substrate) in order to speed up the hydrolysis and ensure high final concentrations of glucose which facilitate downstream processes (fermentation and distillation)(*6-9*).

One approach to reduce the processing cost is to use high substrate (biomass) loads (>15 % w/w), referred to as “high-solids” in the literature, which may reduce cost for water consumption, heating and distillation(*5;10-12*). This approach has been followed in several studies, and seems promising, however, with the main drawback, that the conversion yield decreases linearly with substrate fraction. This relationship has been referred to as the “solid effect” and we will use this term throughout this paper(*12-14*). Several distinct mechanisms may contribute to the solid effect, and previous works have suggested product inhibition(*5;13-18*), mass transfer limitations due to high substrate loadings(*19;20*) or non-productive adsorption/binding to lignin residues or other non-cellulose components such as xylo-oligosaccharides or (poly)-phenols(*21-25*). Several studies (*13;19;26*) have shown that the solid effects occur on many different substrates, ranging from pure cellulose to complex lignocellulose, which rule out adsorption to lignin and release of soluble non-sugar inhibitors as main inhibitory mechanisms at high-solid loadings. Furthermore, if mass transfer due to high substrate loadings is the primary rate limiting factor, a linear decrease is not expected due to the inverse relationship between diffusion and viscosity (Stokes-Einstein equation).This is further supported by the addition of different amounts of cross-linked non-hydrolysable dextrans (i.e. increasing the solid content) in combination with 5 % cellulose which has been shown not to affect cellulose conversion (*27*). Lastly, studies have shown that replacing water with oleyl alcohol only affect enzyme performance slightly, and not to the same extent as seen from the solid effect, which indicate that water activity is not a limiting factor(*13*). The above findings suggest that the solid effect is a generic property associated with the kinetics of cellulase hydrolysis, and not substrate-specific. This, however, does not rule out additional contributions from effects originating from substrate specific properties, including the presence of soluble (poly) phenolics etc.

In this study we investigated the contribution of product inhibition to the solid effect. The experimental approach was to measure product inhibition of a commercial cellulase cocktail (CelluclastTM 1.5 L) directly by addition of glucose to high-solid hydrolysis samples and indirectly by varying the solid/water content (constant mass of solid and enzyme with different volumes of water). Furthermore, the relative strength of glucose and cellobiose inhibition was analyzed through variation of beta-glucosidase activity and discussed in relation inhibition mechanism and enzyme performance under relevant industrial conditions.

**Material and Method**

***Enzymes and substrates***

*Enzymes*

The enzymes used for cellulose hydrolysis were the commercial products CelluclastTM 1.5 L and Novozyme188 ®. CelluclastTM 1.5 L contains a mixture of cellulases (mainly cellobiohydrolases and endoglucosidases) from *T. reesei* and the specific activity was measured to 82 filter paper units (FPU) ml-1 by standard methods(*28;29*). Novozyme 188 mainly contains β-glucosidase from *A. niger* (AnBG) and has a specific activity of 250 cellobiase units ml-1. Celluclast and Novozyme188 solutions were buffer exchanged to 50 mM sodium acetate, 2 mM CaCl2, pH 5 prior to hydrolysis in order to remove excipients, salts and formulation buffer (resulting in a 1.4X dilution). Unless stated otherwise, the buffer exchanged Celluclast was dosed at 10 FPU g-1 cellulose (corrected for dilution due to buffer exchange), and Novozyme188 was dosed 1:4 v/v with respect to celluclast (8 CBU g-1 cellulose).

*Substrate*

Washed ground and sieved pretreated corn stover (WGS-PCS) was used as substrate. In brief, the dry senescent corn stover was pretreated by the National Renewable Energy Laboratory (NREL) by addition of sulfuric acid to 1.5 % w/w (based on total mass of slurry) and heated to 192°C in a Sunds reactor for 3 minutes. After pretreatment, the material was washed, ground and sieved (325 µm mesh size) as described elsewhere(*28*). The composition of the PCS was determined by NREL Laboratory Analytical Procedures (LAP) to be 57.5 % glucan, 7.0 % xylan and 27.2 % lignin (w/w) (acid insoluble). The pH of the PCS suspensions was adjusted to 5 by addition of 0.2 M NaOH. Five mM sodium azide was added to all samples to avoid microbial growth during hydrolysis experiments.

***HPLC analysis***

The sugar concentration (glucose and cellobiose) in the liquid phase was measured by HPLC with RI detection (Agilent 1100 RI, CA, USA). The eluent was 5 mM H2SO4 with a flow rate of 0.6 ml min- 1 and the column temperature was 60 0C. Calibration trials were performed on glucose and cellobiose, and fucose was used as an internal standard. The samples for HPLC analysis were prepared as follows: the hydrolysis experiments were stopped by adding 8 ml 80 0C water to each sample (1-6 g depending on solid content) and heated to 95 0C for 20 minutes. During this inactivation time, samples were vigorously shaken to ensure efficient sugar extraction. The above extraction step were repeated with 8 ml 50 % ethanol, as this has been shown to improve extraction efficiency(*30*). The supernatants from the two extraction steps were pooled and reduced to approximately 4 g (measured by weighing at room temperature) by evaporation at 80 0C. The samples were filtered through a 0.45 µm nylon filter, mixed with internal standard and mounted on the HPLC auto sampler.

***Variation of water content***

Twenty gram 35.3 % (w/w) PCS was transferred to a glass beaker on ice. Sodium azide (5 mM) and enzyme (Celluclast + Novozym188) and substrate were mixed on ice using a metal spatula for 2 minutes to a final concentration of 10 FPU g-1 cellulose. The precise mass of ~ 1 g aliquots of 33.3 % (slightly diluted due to addition of enzyme and azide solution) biomass were determined and each sample was added buffer (0-5 ml) to produce a series of samples with solid contents between 7 and 33% in 15 ml falcon tubes with a final mass between 1 and 6 g depending on volume of added buffer. This resulted in samples with fixed amount of solid and enzyme, but with decreasing solid fraction (and increasing liquid fraction). After mixing the samples were immediately mounted on a rotator wheel (2 RPM) at 50 0C for 20 h hydrolysis and then thermally inactivated (95 0C 20 min.) and analyzed by HPLC as described above.

***Variation of initial glucose concentrations***

Twenty gram 35.3 % (w/w) PCS was mixed with enzyme (same dosage as above) on ice and weight out in 1 g portions as described above. Each sample was mixed with increasing amount of glucose by addition of 0.26 ml (0-2M) glucose. This resulted in samples with increasing initial glucose concentrations (0-562 mM) with a fixed solid fraction of 27 % (w/w). Hydrolysis for 20 h and analysis were conducted as described above.

*Moderate/high beta-glucosidase concentrations*

To investigate the effect of accumulated cellobiose during enzymatic hydrolysis, experiments with moderate (1/4 volume of Novozyme188 relative to Celluclast, resulting in 8 CBU g-1 cellulose) and excess (980 CBU g-1 cellulose equivalent to 4 mg g-1 cellulose of purified AnBG) BG were performed*”.*

These experiments were designed as described above, keeping enzyme (Celluclast) and solid content fixed, while varying the water content. Thus, in case of moderate BG concentration, a build-up of cellobiose is expected as the liquid volume decreases, while the cellobiose concentration is negligible (measured to be < 2.5 mM) at all liquid fractions in case of excess BG.

***Calculations***

The calculations of conversion in high-solid samples are often complicated by the change in volume of the solid and liquid fractions as well as density change of the liquid phase during hydrolysis. This may lead to overestimation of conversion by up to 35 % as noted by (*26*). In the present work this issue was avoided by the addition of 16 ml extraction solution as described above, which eliminates the potential error from underestimation of the liquid hydrolysate volume in high solid samples. Conversion was calculated as the degree of solubilization using the ratio between glucosyl units in the supernatant (nGlu, nCel\*2), and the theoretical number of glucosyl units in the biomass (eq 1).

Conversion = ((nGlu + nCel\*2) / (mS\*XSolid\*XCellulose)/MGlucosyl)\*100 (1)

Where mS denotes sample mass of biomass/enzyme slurries, Xsolid is the weight fraction of solids in substrate/enzyme slurry, XCellulose is weight fraction of cellulose in the solids, and MGlucosyl is the molar mass of glucosyl units in cellulose (162 g mol-1).

The concentration of glucose and cellobiose in the hydrosylate was calculated based on nGlu and nCel as above and estimation of the liquid volume in the hydrosylate sample. The liquid volume was corrected for liquefaction by adding the volume increase from released glucose and cellobiose, using the density of 1.52 g/ml corresponding to the density of pure glucose (eq 2). Control calculations have shown that this approximation gives less than 1 % error in calculated volume (using tabulated densities of known glucose/water weight fractions).

Liquid volume = (mS\*XW)/1 g ml-1 + VW + (mGlu+mCel)/1.52 g ml-1 (2)

Where XW is the initial water fraction of the biomass/enzyme slurry and VW is the volume of added buffer and 1.52 g/ml is the density of pure glucose.Since water is a substrate for hydrolysis, the water consumption was calculated, however, this only account for less than 1 % of the liquid volume (at the highest solid content) and was therefore not included in eq. 2.

**Results and discussion**

*Relationship between the solid-effect and product concentration*

Increasing the solid fraction, by reducing the water content, in samples with fixed amount of biomass and enzyme resulted in a linear decrease in yield (Fig 1A) after 20 h hydrolysis. This is a clear signature of the solid effect as it is also observed for other substrates in the literature(*13;19;26*). Thus, the conversion from 20 h hydrolysis at 50 0C fell from 65 % to 35 % going from 7 % to 33 % solid (panel A). These values are comparable to the solid effect for filter paper under comparable experimental conditions (dose, temperature and hydrolysis time)(*13*). It should be noted, that the solid effect may depend on hydrolysis time, with a tendency to almost disappear at extended hydrolysis times where conversion reach the theoretical maximum at the highest solid loadings (*14*).



Fig. 1

Panel 1B show the concentration of glucose and cellobiose as a function of solid content in the same samples as panel 1A. As seen from the figure, both glucose and cellobiose concentration increased linearly with solid content. While glucose may be considered an end product of the enzymatic hydrolysis, cellobiose is an intermediate, whose concentration depends on the BG activity. The increase in glucose is expected due to the reduction in water volume with increasing solids. The increase in cellobiose concentration might be attributed to decreased BG activity caused by glucose inhibition, as also shown in previous studies(*31-34*). Since both sugars are known to inhibit the cellulase complex in *Tricoderma reesei*, the decrease in conversion with increasing solids might be attributed to product inhibition by both sugars.

To test whether product inhibition is important within the concentration range observed in Fig. 1B, a new set of experiments were performed, where glucose was added initially to 33 % (w/w) PCS with enzyme on ice. As seen from Fig. 2, increasing the initial glucose concentration from 0 to 562 mM, which corresponds to the range of accumulated glucose concentration in Fig. 1B, resulted in a linear decrease in hydrolysis yield at a constant solid content of 27 % (w/w). The insert show that cellobiose concentration increases linearly with the initial glucose concentration. As mentioned above, this probably reflects BG inhibition.



Fig. 2

Based on Fig. 1 and 2, it is concluded that; i) Conversion decrease and accumulated product concentration increases linearly with solid fraction ii) Addition of glucose in amounts comparable to those produced by the hydrolysis in high-solid samples, results in a linear decrease in conversion at a fixed (high) solid load. These conclusions support the interpretation that product inhibition contributes significantly to the solid effect.

Further insight into the importance of product inhibition for the solid effect might be gained by plotting conversion against cumulated glucose concentration in the two experiments presented in Fig 1 and 2. The strength of this analysis is the correlation between conversion and product concentration when two distinct independent variables are changed, *i.e.* water content and initial glucose concentration. Hence in case of the “water variation experiment”, conversion is plotted as a function of accumulated glucose, whereas in case of the “initial glucose experiments” (constant solid fraction), conversion is plotted against total glucose (added +produced). As seen from Fig. 3A the two plots fall on a continuous curve which was fitted using an exponential decay function for both glucose and cellobiose.



Fig. 3

The results from Fig. 3 indicate that the major contributor to the solid effect is product inhibition. If other parameters coupled to the water content were important, these would not fall on the extension of results from the water experiment as it is indeed seen for both glucose and cellobiose (Fig. 3.). Thus, parameters such as viscosity changes, substrate hydration, substrate availability or “other inhibitors” (as soluble phenols, xylose etc.) which are all expected to depend on water content, do not seem to be the main parameters controlling the solid effect, at least not for the substrate and concentration ranges examined here. It should be noted, however, that the current substrate has been washed following the pretreatment, which might reduce the presence of soluble inhibitors as xylo-oligomers and (poly) phenols(*22*) compared to unwashed substrates. The above conclusion is in agreement with recent studies by Selig et al. who showed that addition of cross-linked dextrans to cellulose hydrolysis samples did not affect conversion, which would be expected if viscosity and diffusion of cellulases are the rate limiting factors under high-solid conditions(*27*). Hence, even though it has been shown, that the effective diffusion coefficient of a model protein (BSA) is reduced under high-solid conditions(*20*), this effect do not appear to be rate limiting for hydrolysis.

The actual mechanism behind glucose inhibition has been debated in the literature(*18;20;27;35*). Since the apparent IC(50) values (50 % reduction in activity) are in the order of several hundred mili molar glucose, interactions are suggested to be weak as compared to classical competitive inhibition where the inhibitor binds tightly to the active site. This view is supported by recent studies suggesting that the inhibitory effect of glucose are primarily governed by weak interactions with water which increase the water restriction (measured as a reduction in water relaxation time, T2, by low field NMR) in the hydrosylate(20;27;35). This is further supported by inhibitory effects from other mono-saccharides as mannose, galactose and xylose where a correlation was observed between T2 and hydrolysis yield (35). Furthermore, it is well know that addition of so-called cosmotropic osmolytes (sugars, poly-alcohols and certain amino acids) reduce the catalytic activity of a number of enzymes through so-called preferential interactions and crowding(36). Hence the inhibitory effect of glucose shown in Fig. 2 and 3A may originate from a combination of weak interactions with both enzymes and water resulting in high IC(50) values, which non or the less give rise to the solid effect, and therefore also are of significant importance for the industrial application of cellulases.

*Estimation of relative inhibition by glucose and cellobiose*

The data in Fig. 3 does not give any information of the relative inhibitory strength of glucose and cellobiose. Rather, it only suggests that the yield decreases with both products. As mentioned above, cellobiose may be considered an intermediate, whose concentration is regulated by BG activity. Hence, one approach to control the cellobiose concentration is to change the BG activity, and ultimately effects of cellobiose may be eliminated altogether at high BG doses (*31;32;37*). We applied this approach by performing experiments as in Fig. 1 with respectively high and moderate BG activity. Hence, in case of moderate BG activity, cellobiose is accumulated proportionally to the solid fraction (as seen in Fig. 1B), whereas the cellobiose concentration is kept low (measured to be < 2.5 mM) at high BG concentration. This enabled us to single out inhibitory effect of glucose and cellobiose, respectively. This analysis is presented in Fig. 4, where the relative decrease in conversion (C/C0) is plotted against respectively the concentration of glucose (panel A) and cellobiose (panel B). In case of glucose, data points for high BG concentration are used, since the cellobiose concentration was kept low (< 2.5 mM). Here C0 was determined by extrapolation to zero glucose. For cellobiose (panel B), the percentage drop in yield was determined by dividing the conversion at moderate BG with high BG activity at identical glucose concentrations (see insert in panel B). Here C0 in the conversion at high BG activity, i.e. negligible cellobiose.



Fig. 4

As seen, increasing concentration of both inhibitors results in a linear decrease in relative conversion, but the slopes are quite different. Thus, glucose inhibition was estimated to have IC(50) (50 % reduction in activity) of 727 mM and cellobiobiose IC(50) around 44 mM. Both of these values were obtained by linearly extrapolating the data in Fig. 4 A and B to C/C0=0.5. In other words, cellobiose appears to be ~15 times more inhibitory than glucose on a molar scale. The IC(50) values for the cellulase complex found in the current work is in reasonable agreement with a recent study on glucose and cellobiose inhibition of mono-component cellulases hydrolyzing regenerated amorphous cellulose(*38*). For example Cel7A (CBH 1), which is the most abundant cellulase in *Trichoderma reesei* cellulase complex, was found to have IC(50) of 19 mM and 400 mM for cellobiose and glucose respectably. Cel6A (CBH II) was found to have IC50 >80 mM for cellobiose and 140 mM for glucose. Cel5A (EG I) and Cel7B (EGII) are also inhibited by both inhibitors within the same order of magnitude(*38*). It should be noted that other studies have found 1-2 order of magnitude lower IC(50) values for cellobiose in case of Cel7A(*39;40*). This difference most likely reflects differences in experimental conditions, especially low substrate concentration, as compared to this study and the study of Murphy et al. (*38*). IC(50) should be considered an empirical parameter, that depends on the experimental conditions as substrate type (affinity to substrate vs inhibitor), concentration (apparent IC(50) increase with substrate concentration), hydrolysis time and temperature as also noted elsewhere(*40*).

Based on the findings above, glucose may inhibit both cellobiohydrolases and endoglucanases directly as is the case when excess BG is added or indirectly through inhibition of BG which results in increasing cellobiose concentrations (Fig. 5).

****

Fig. 5

Considering the relative strength of glucose and cellobiose inhibition (cellobiose ~ 15X more inhibitory) in relation to observed concentrations industrially (~1M glucose, ~10 mM cellobiose), both inhibitory mechanisms are important for the final yield in high-solid hydrolysis.

In summary, the current work concludes that product inhibition from both glucose and cellobiose is the main cause of the solid effect under the given experimental conditions. The estimated inhibition constants of glucose and cellobiose are in reasonable agreement with literature values of both mono-component cellulases and cellulase cocktails. These findings suggest that factors as viscosity, substrate hydration, substrate availability or “other inhibitors” (as soluble phenols, xylose etc.) which are all expected to depend on water content, do not seem to contribute significantly to the solid effect, at least not for the substrate and concentration ranges examined here.

**Fig. 1: Effect of increasing solid fraction on cellulose conversion (A) and accumulated product concentration, glucose and cellobiose (B), after 20 h hydrolysis at 500C.** Panel A: samples of 1 gram of 35 % WGS-PCS was dosed with 10 FPU g-1 cellulose on ice and mixed with 0-4 ml buffer to obtain solid fractions from 6-33 %. Panel B: Accumulated glucose (green) and cellobiose (blue) concentrations after 20 h hydrolysis.

**Fig. 2: Effect of initial glucose addition on cellulose conversion. Insert: Accumulated cellobiose as a function of initial glucose concentration.** 1 g. of 35 % PCS were was dosed with 10 FPU g-1 cellulose on ice and mixed with 0.26 ml 0-2 M glucose, resulting in initial glucose concentrations between 0-562 mM at a fixed solid content of 27 % (w/w). Hydrolysis was performed at 500C for 20h.

**Fig. 3: Combination of “water” (Fig. 1) and “initial glucose” (Fig. 2).** Panel A: Conversion is plotted against accumulated glucose concentration for the “water” (solid circles) and “initial glucose” (open squares) experiments. An exponential decay function provided the best fit to data (R2= 0.97). Panel B: Conversion as a function of accumulated cellobiose in the “water” and “initial glucose experiments”. An exponential decay function provided the best fit to the data (R2= 0.95).

**Fig. 4: Isolation of inhibitory effect from glucose and cellobiose through variation of beta-glucosidase activity.** Panel A: Isolation of the inhibitory effect of glucose. C/C0 denotes conversion at a given glucose concentration (C) divided by the conversion in the absence of glucose, estimated by extrapolation to zero glucose (data point from high BG experiment). Panel B: Isolation of the inhibitory effect of cellobiose. C/C0 is calculated by divition of conversion at a given cellobiose concentration (moderate BG) with the conversion in the absence of cellobiose (high BG) but at the same glucose concentration (see insert). Insert: Conversion as a function of accumulated glucose at high BG activity (closed circles) and moderate BG activity (open squares).

**Fig. 5: Schematic drawing of proposed inhibition mechanism in high-solid saccharification.** The relative importance of the “direct” and “indirect” glucose inhibition depends on BG activity.

**References**

 **1. Gomez, L. D., Steele-King, C. G., and McQueen-Mason, S. J. (2008) *New Phytologist* *178*, 473-485.**

 **2. Galbe, M. and Zacchi, G. (2002) *Applied Microbiology and Biotechnology* *59*, 618-628.**

 **3. Lynd, L. R., Weimer, P. J., van Zyl, W. H., and Pretorius, I. S. (2002) *Microbiology and Molecular Biology Reviews* *66*, 739.**

 **4. Zhang, Y., Himmel, M. E., and Mielenz, J. R. (2006) *Biotechnology Advances* *24*, 452-481.**

 **5. Jorgensen, H., Kristensen, J. B., and Felby, C. (2007) *Biofuels Bioproducts & Biorefining-Biofpr* *1*, 119-134.**

 **6. Andric, P., Meyer, A. S., Jensen, P. A., and Dam-Johansen, K. (2010) *Biotechnology Advances* *28*, 308-324.**

 **7. Ohgren, K., Vehmaanpera, J., Siika-Aho, M., Galbe, M., Viikari, L., and Zacchi, G. (2007) *Enzyme and Microbial Technology* *40*, 607-613.**

 **8. Heinzelman, P., Snow, C. D., Wu, I., Nguyen, C., Villalobos, A., Govindarajan, S., Minshull, J., and Arnold, F. H. (2009) *Proceedings of the National Academy of Sciences of the United States of America* *106*, 5610-5615.**

 **9. Andric, P., Meyer, A. S., Jensen, P. A., and Dam-Johansen, K. (2010) *Biotechnology Advances* *28*, 407-425.**

 **10. Hodge, D. B., Karim, M., Schell, D. J., and McMillan, J. D. (2009) *Applied Biochemistry and Biotechnology* *152*, 88-107.**

 **11. Wingren, A., Galbe, M., and Zacchi, G. (2003) *Biotechnology Progress* *19*, 1109-1117.**

 **12. Humbird, D., Mohagheghi, A., Dowe, N., and Schell, D. J. (2010) *Biotechnology Progress* *26*, 1245-1251.**

 **13. Kristensen, J. B., Felby, C., and Jorgensen, H. (2009) *Biotechnology for Biofuels* *2*.**

 **14. Hodge, D. B., Karim, M., Schell, D. J., and McMillan, J. D. (2008) *Bioresource Technology* *99*, 8940-8948.**

 **15. Puri, D. J., Heaven, S., and Banks, C. J. (2013) *Biotechnology for Biofuels* *6*.**

 **16. Holtzapple, M., Cognata, M., Shu, Y., and Hendrickson, C. (1990) *Biotechnology and Bioengineering* *36*, 275-287.**

 **17. Du, F., Wolger, E., Wallace, L., Liu, A., Kaper, T., and Kelemen, B. (2010) *Applied Biochemistry and Biotechnology* *161*, 313-317.**

 **18. Xiao, Z. Z., Zhang, X., Gregg, D. J., and Saddler, J. N. (2004) *Applied Biochemistry and Biotechnology* *113*, 1115-1126.**

 **19. Modenbach, A. A. and Nokes, S. E. (2013) *Biomass & Bioenergy* *56*, 526-544.**

 **20. Roberts, K. M., Lavenson, D. M., Tozzi, E. J., McCarthy, M. J., and Jeoh, T. (2011) *Cellulose* *18*, 759-773.**

 **21. Olsen, S., Bohlin, C., Murphy, L., Borch, K., McFarland, K., Sweeny, M., and Westh, P. (2011) *Enzyme and Microbial Technology* *49*, 353-359.**

 **22. Ximenes, E., Kim, Y., Mosier, N., Dien, B., and Ladisch, M. (2010) *Enzyme and Microbial Technology* *46*, 170-176.**

 **23. Baumann, M. J., Borch, K., and Westh, P. (2011) *Biotechnology for Biofuels* *4*.**

 **24. Kim, Y., Ximenes, E., Mosier, N. S., and Ladisch, M. R. (2011) *Enzyme and Microbial Technology* *48*, 408-415.**

 **25. Qing, Q., Yang, B., and Wyman, C. E. (2010) *Bioresource Technology* *101*, 9624-9630.**

 **26. Kristensen, J. B., Felby, C., and Jorgensen, H. (2009) *Applied Biochemistry and Biotechnology* *156*, 557-562.**

 **27. Selig, M. J., Hsieh, C. W. C., Thygesen, L. G., Himmel, M. E., Felby, C., and Decker, S. R. (2012) *Biotechnology Progress* *28*, 1478-1490.**

 **28. Murphy, L., Borch, K., McFarland, K., Bohlin, C., and Westh, P. (2010) *Enzyme and Microbial Technology* *46*, 141-146.**

 **29. Ghose, T. K. (1987) *Pure and Applied Chemistry* *59*, 257-268.**

 **30. Olsen, S. N., Lumby, E., McFarland, K., Borch, K., and Westh, P. (2011) *Applied Biochemistry and Biotechnology* *163*, 626-635.**

 **31. Berlin, A., Maximenko, V., Gilkes, N., and Saddler, J. (2007) *Biotechnology and Bioengineering* *97*, 287-296.**

 **32. O'Dwyer, J. P., Zhu, L., Granda, C. B., and Holtzapple, M. T. (2007) *Bioresource Technology* *98*, 2969-2977.**

 **33. Bohlin, C., Olsen, S. N., Morant, M. D., Patkar, S., Borch, K., and Westh, P. (2010) *Biotechnology and Bioengineering* *107*, 943-952.**

 **34. Singhania, R. R., Patel, A. K., Sukumaran, R. K., Larroche, C., and Pandey, A. (2013) *Bioresource Technology* *127*, 500-507.**

 **35. Hsieh, C. w., Cannella, D., Jorgensen, H., Felby, C., and Thygesen, L. G. (2014) *Journal of Agricultural and Food Chemistry* *62*, 3800-3805.**

 **36. Olsen, S., Ramlov, H., and Westh, P. (2007) *Comparative Biochemistry and Physiology A-Molecular & Integrative Physiology* *148*, 339-345.**

 **37. Kumar, R. and Wyman, C. (2009) *Biotechnology and Bioengineering* *102*, 457-467.**

 **38. Murphy, L., Bohlin, C., Baumann, M. J., Olsen, S. N., Sorensen, T. H., Anderson, L., Borch, K., and Westh, P. (2013) *Enzyme and Microbial Technology* *52*, 163-169.**

 **39. Gruno, M., Valjamae, P., Pettersson, G., and Johansson, G. (2004) *Biotechnology and Bioengineering* *86*, 503-511.**

 **40. Teugjas, H. and Vaeljamaee, P. (2013) *Biotechnology for Biofuels* *6*.**