HIGH SOLID AND LOW ENZYME LOADING BASED SACCHARIFICATION OF AGRICULTURAL BIOMASS

Yu Zhang, Yun-Yun Liu, Jing-Liang Xu,* Zhen-Hong Yuan,* Wei Qi, Xin-Shu Zhuang, and Min-Chao He

Two agricultural biomass materials, namely wheat straw and sugarcane bagasse, were pretreated with NaOH and then used as substrates for enzymatic saccharification. After the pretreatment, the increase in glucan content and the decrease in lignin content were more than 65%, while less than 20% increase occurred in xylan content. The enzymatic saccharification was initiated with solid loading 9% (w/v), and then 8%, 7% and 6% (w/v) solid was fed at 8, 24, and 48 h, respectively. The final enzyme solid loading was 9.60 FPU/g solid and 30% (w/v), respectively. At 144 h, the produced glucose, xylose, and reducing sugar concentrations for wheat straw were 81.88, 20.30, and 115.25 g/L, respectively, and for sugarcane bagasse they were 125.97, 8.66, and 169.50 g/L, respectively. The final conversions of wheat straw and sugarcane bagasse were 34.57% and 50.85%, respectively. SEM images showed that the surface structure of the two materials changed a lot via alkali-pretreatment and enzymatic hydrolysis. In summary, a high concentration sugar is produced from the two agricultural biomass materials by high solid and low enzyme loading. Compared to wheat straw, sugarcane bagasse is more suitable for use in sugar production.

Keywords: Cellulase; Fermentable sugar; High solid loading; Enzymatic saccharification; Biomass

*Contact information: Key Laboratory of Renewable Energy and Gas Hydrate, Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences, Guangzhou 510640, P.R. China; * Corresponding author: xjl@ms.giec.ac.cn, yuanzh@ms.giec.ac.cn*

INTRODUCTION

The current dependency on fossil resources for energy and chemicals production will not be able to ensure the sustainable development of human society in the future, and this situation puts tremendous focus on finding alternative resources (Jørgensen et al. 2007). In this respect, lignocellulose, as in the form of agricultural biomass, will be a better choice due to its renewability, abundance, and low cost. How to make full use of the lignocellulose resource has become an issue of concern around the world. Conversion of lignocellulose to fermentable sugar (saccharification) is one of the most common routes for lignocellulose utilization, and many liquid fuels and chemicals including ethanol, butanol, and organic acids could be produced from the sugar (Casler et al. 2009).

Obviously, saccharification plays an important part in industrial production of these fuels and chemicals. In order to obtain high-efficiency saccharification, hydrolytic enzymes including cellulase are always involved in the process (Zhang et al. 2006). It is critically important to achieve a high concentration of sugar before the subsequent conversion. High solid loading-based saccharification is a direct and convenient technique to

obtain a high concentration of sugar (Hodge et al. 2009). Besides, there are other benefits: lower capital costs due to reduced volume; lower operating costs due to less energy required for heating and cooling; and reduced disposal and treatment costs due to lower water usage. However, a high concentration of substrate increases the viscosity of the saccharification system, leading to a decline in hydrolysis efficiency and an increase in power consumption for stirring (Kristensen et al. 2009; Lu et al. 2010; Olsen et al. 2011; Pristavka et al. 2000; Zambare et al. 2011). Under this circumstance, high enzyme loading is required in order to achieve a high concentration of sugar. However, the hydrolytic enzyme for lignocellulose saccharification is costly (Zhang et al. 2006). To render the process economically viable, a fed batch system has been proposed to reduce enzyme loading. It has been reported that 175 g/L glucose could be produced from 30% (w/v) corn stover by three fed-batches and low enzyme loading (Yang et al. 2010). However, the pretreatment was very complicated, which also greatly increased the conversion cost.

In this study, two agricultural biomass materials, namely wheat straw and sugarcane bagasse, were used as substrates for high solid and low enzyme loading-based saccharification after alkali-pretreatment.

EXPERIMENTAL

Materials

The enzyme used for hydrolysis was Accellerase® 1500, provided by Genencor Co., Ltd. (Wuxi, China). The activity of the cellulase was 43.21 FPU/mL (FPU was the activity unit when filter paper was used as the enzymatic substrate), measured by the description of IUPAC (International Union of Pure and Applied Chemistry) (Ghose 1987). For the assay procedures described here, 2.0 mg of reducing sugars (glucose equivalents) were produced from a piece of Whatman grade No. 1 filter paper (1 × 6 cm, about 50 mg) at specified dilutions of enzyme solution.

Wheat straw was obtained from a farm in a local harvest (Wuhu, China). Sugarcane bagasse provided by Guangxi Fenghao Sugar Co., Ltd (Chongzuo, China), was peeled. The two materials were milled and screened, and the fraction between 18 and 40 mesh was used for alkali-pretreatment.

Alkali Pretreatment

Wheat straw and sugarcane bagasse were pretreated by 0.5 M NaOH in a round-bottom flask at 80 ^{o}C and 120 rpm (solid-liquid ratio is 1:20). After 6 h, the two pretreated materials were washed with hot water until the washing liquid was neutral and no sugar could be detected from the liquid. The obtained residues were dried at 80 ^{o}C, and then they were used as solid substrates for subsequent enzymatic hydrolysis.

Enzymatic Hydrolysis

High solid based enzymatic hydrolysis was initiated with 9% (w/v) solid loading and 32 FPU/g solid, and then 8% (w/v), 7% (w/v), and 6% (w/v) solid was fed consecu-

tively at 8 h, 24 h, and 48 h, respectively. The letters w and v represented the solid mass (g) and the liquid volume (mL), respectively.

Only pure solid was fed at each desired time, and no any buffer solution or enzyme was added during the feeding. Therefore, the final solid and enzyme loading was 30% (w/v) and 9.6 FPU/g solid, respectively. All the reactions were carried out at pH 5.0 (0.2 M acetate buffer), 50 ^{o}C, and 120 rpm. At each desired time, a sample solution was taken out and centrifuged at 4000 rpm and 4 ^{o}C for 5 min. The obtained supernatant was kept at 80 ^{o}C for 10 min and then used for sugar assay. The conversion ratios of glucan, xylan and raw material were calculated as follows (Zhu et al. 2011),

$$\text{Glucan conversion ratio}\,(\%) = \frac{\text{Produced glucose amount} \times 0.90}{\text{Glucan amount in enzymatic substrate}} \times 100$$

$$\text{Xylan conversion ratio}\,(\%) = \frac{\text{Produced xylose amount} \times 0.88}{\text{Xylan amount in enzymatic substrate}} \times 100 \quad (1)$$

$$\text{Raw material conversion ratio}\,(\%) = \frac{\text{Produced reducing sugars amount} \times 0.90}{\text{Raw material amount}} \times 100$$

where the used raw material (enzymatic substrate) concentrations at/after 8, 24, and 48 h were 9, 17, and 24 g/L, respectively.

Sugar Assay

Reducing sugars concentration was determined by the DNS (3,5-dinitrosalicylic acid) method (Ghose 1987).

Glucose and xylose concentrations were determined by the HPLC (high-performance liquid chromatography) Waters 2695 system consisting of a Waters 600E system controller, a Waters 717 automatic sampler, a Waters 2414 differential refractometer, and a Shodex sugar SP-0810 column. The mobile phase was 0.005 mol/L H_2SO_4 at a flow rate of 0.5 mL/min. The column temperature was 80 ^{o}C. The injected sample volume was 10 μL. Standard samples and hydrolyzed samples were filtrated by 0.45 μm filter before analysis.

Composition Assay

Glucan, xylan, and Klason lignin content in raw, alkali-pretreated, and enzyme hydrolyzed wheat straw/sugarcane bagasse were analyzed according to the standard Laboratory Analytical Procedures (LAP) provided by the National Renewable Energy Laboratory (NREL).

SEM Assay

The surface morphology and characteristics of the different residual solid were analyzed by SEM (Scanning Electron Microscope). Images were taken using the model JEOL JSM-6360LV SEM and performed at a beam accelerating voltage of 10 kV. Digital images were captured using 1280×960 resolution and 160 s dwell time.

RESULTS AND DISCUSSION

Composition Changes from Alkali Pretreatment

To facilitate rapid and efficient hydrolysis of carbohydrates, the raw materials must be pretreated. Lignin acts as a protective physical barrier to cellulase/ hemicellulase attack, and it should be removed before enzymatic hydrolysis. Toward this aim, alkali-pretreatment was carried out. As shown in Table 1, alkali-pretreatment removed the major proportion of lignin of wheat straw/sugarcane bagasse, and a little lignin remained in the pretreated materials. Klason lignin content in wheat straw and sugarcane bagasse was reduced by 77.16% and 67.46%, respectively. On the other hand, glucan content in the two materials was increased a lot via alkali-pretreatment. The increase of glucan content in wheat straw was 75.46%, and in sugarcane bagasse it was 66.57%. By contrast, little loss (less than 20% decrease) in xylan content of the two materials was suffered as a result of the pretreatment. It was concluded that alkali-pretreatment could effectively remove the lignin and retain most of the cellulose. The change helped to facilitate subsequent enzymatic saccharification operating at high solid loading.

Table 1. Composition Analysis of Raw, Alkali-Pretreated, and Hydrolyzed Wheat Straw/Sugarcane Bagasse[a]

	Wheat straw (%)			Sugarcane bagasse (%)		
	Raw	Alkali-pretreated	Enzyme hydrolyzed	Raw	Alkali-pretreated	Enzyme hydrolyzed
Glucan	35.60	62.46	30.33	41.20	68.63	25.11
Xylan	24.67	27.29	12.75	20.46	24.33	15.69
Klason lignin	18.66	4.26	14.80	20.04	6.52	10.19

[a] Composition content in pretreated and hydrolyzed solids was relative pretreated and hydrolyzed solids, respectively, other than the raw solids.

Fed Batch Based Enzymatic Hydrolysis

Figure 1 shows the effect of time on the produced sugar concentrations from enzymatic hydrolysis of pretreated wheat straw and sugarcane bagasse. Glucose, xylose, and reducing sugar concentrations increased with the increase of hydrolytic time. For both wheat straw and sugarcane bagasse, the hydrolysis rates were maximum at the initial stage, and they gradually became smaller and smaller as time increased. With this situation in view, fed batch was carried out because high concentration substrate could improve enzyme saturation and help relieve the rate slowdown. The slowdown of the hydrolytic rate during the whole hydrolytic process was mainly caused by enzyme deactivation, products inhibition, and the decrease of utilizable substrate. More than 90% of total hydrolysates were released during the first 96 h (48 h after the last fed), and hydrolysates increased relatively little during the next 48 h. At 144 h, the produced glucose, xylose, and reducing sugar concentrations for wheat straw were 81.99, 20.30, and 115.25 g/L, respectively. For sugarcane bagasse they were 125.97, 8.66, and 169.50 g/L, respectively. Although the produced glucose and reducing sugar concentrations from sugarcane wheat straw were significantly higher than those from bagasse, the produced xylose concentration was lower. This result may be caused by higher glucan and lower xylan content in pretreated sugarcane bagasse than that in pretreated wheat straw.

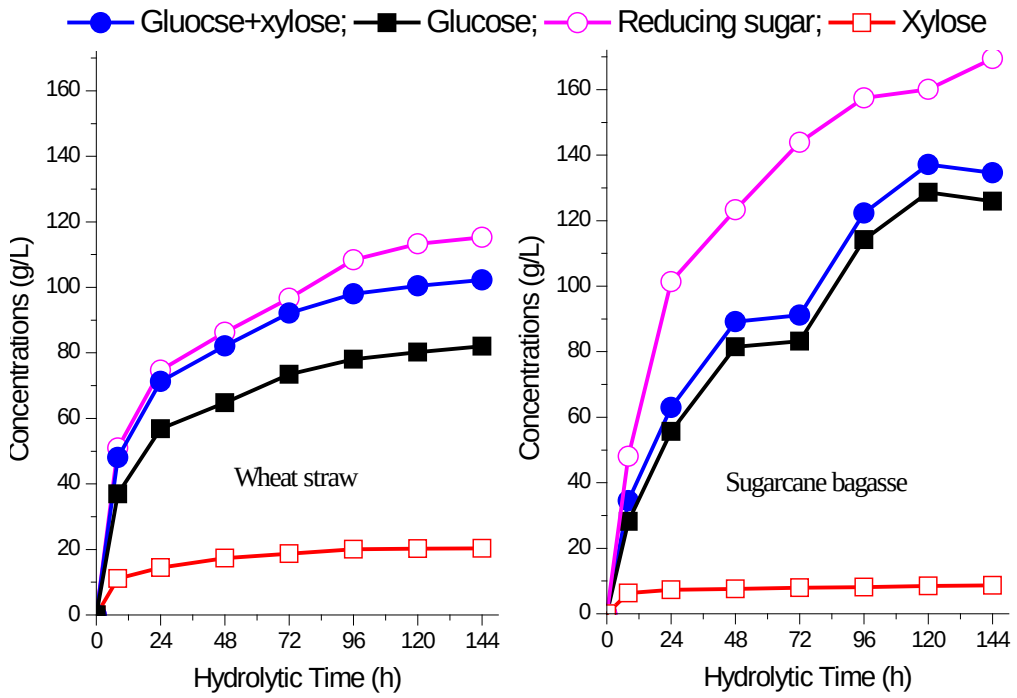

Fig.1. Hydrolysates concentrations from enzymatic hydrolysis of alkali-pretreated wheat straw and sugarcane bagasse at different times

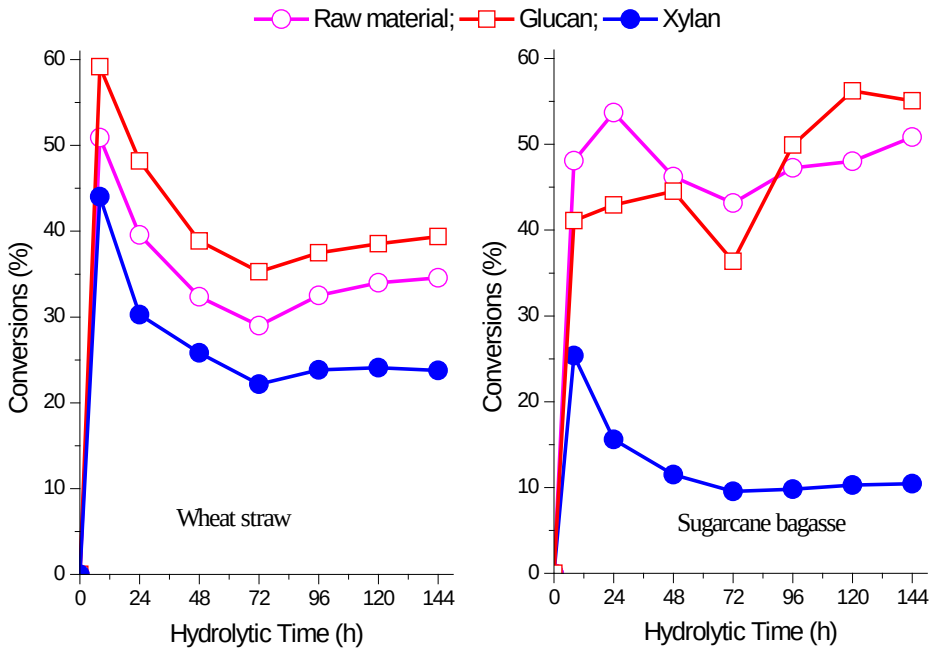

Fig. 2. Glucan, xylan, and raw material conversions during the hydrolysis of alkali-pretreated wheat straw (a) and sugarcane bagasse (b). The used solid concentrations for calculating conversions at/after 8, 24 and 48 h were 9, 17, and 24 g/L, respectively.

The conversions of glucan, xylan, and raw material during the enzymatic hydrolysis are shown in Fig. 2. For wheat straw, the conversions reached their maximum at the first feed, and then they gradually decreased as a result of subsequent feedings. After 72 h, the conversions increased slightly, and the final conversions of glucan, xylan, and raw material were 39.38%, 23.78%, and 34.57%, respectively. A similar trend of conversions occurred during saccharification of sugarcane bagasse. Distinctly, a sharp decrease and increase in glucan conversion was evident from 48 to 72 h and from 72 to 96 h, respectively. At 42 h, the last feed made the viscosity of the saccharification system become very high, so the hydrolytic speed was very large. As the hydrolysis proceeded, the viscosity gradually decreased and the hydrolytic speed had begun to increase after 72 h. After 144 h, the final conversion of glucan, xylan, and raw material was 55.07%, 10.44%, and 50.85%, respectively.

SEM

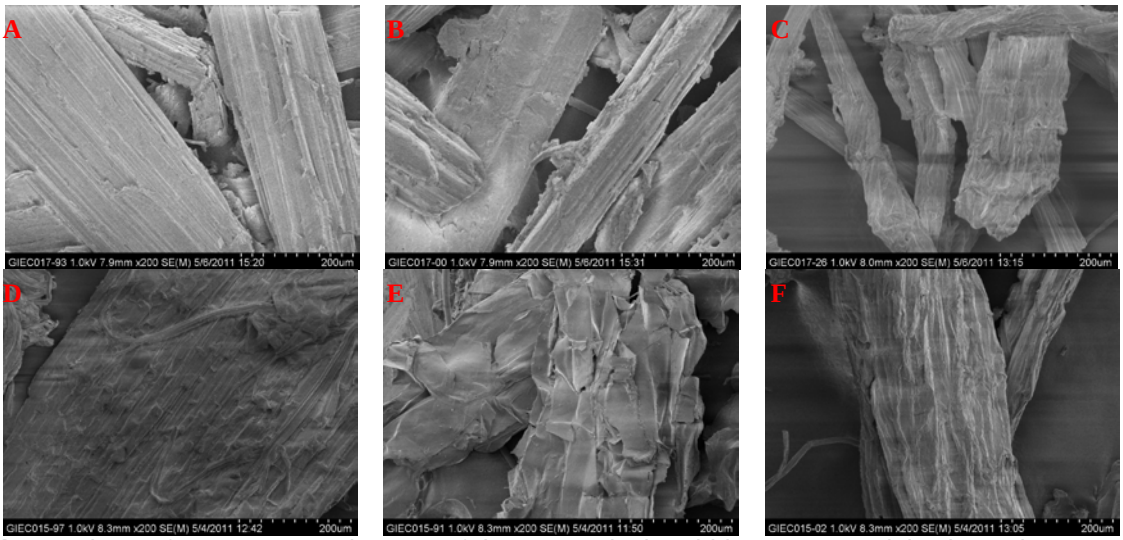

Fig. 3. The surface structure changes of the two agricultural biomass materials shown in SEM pictures (200 ×). A, B, and C represent raw, alkali-pretreated, and enzyme-hydrolyzed wheat straw, respectively; D, E, and F represents raw, alkali-pretreated, and enzyme-hydrolyzed sugarcane bagasse, respectively.

Figure 3 shows the surface physical structure changes of raw, alkali-pretreated, and enzyme hydrolyzed wheat straw/sugarcane bagasse. The structure of raw materials was very compact, and no fragmentation could be seen in the SEM pictures. After alkali-pretreatment, their physical structure was broken seriously, and some smaller fragments were produced as a result of lignin removal. The surface also became rough and loose via the pretreatment, and showed many grooves. These changes could help in subsequent enzymatic saccharification by allowing for hydrolytic enzymes to penetrate the substrate. After 144 h enzymatic hydrolysis, the surface of wheat straw/sugarcane bagasse became smooth and compact again because the rough and loose fragments were consumed by enzymatic hydrolysis.

DISSCUSSION

Reports involving high solid loading based saccharification of wheat straw and sugarcane bagasse have not yet been seen. However, it has been reported that other lignocellulosic materials have been used as high concentrate substrates for saccharification (Table 2).

Table 2. Sugar Concentrations Produced from High Solid Loading-Based Saccharification of Various Cellulosic Materials

Reference	Substrate	Pretreatment	Solid loading (w/v)	Enzyme loading (FPU/g solid)	Hydrolytic time (h)	Produced sugar concentration (g/L)
Olsen et al. (2011)	Partially crystalline cellulose	No	41%[a]	78[a]	18	≈ 80[a] (glucose)
	Corn stover	sulfuric acid		144[a]		≈ 70[a] (glucose)
Yang et al. (2010)	Corn stover	Steam-exploded and NAOH-H_2O_2	30%[b]	8	144	220 (reducing sugar), 175 (glucose), 22 (cellobiose), 20 (xylose)
Lu et al. (2010)	Corn stover	Steam-exploded	30%	20	96	103.3 (glucose)
Kristensen et al. (2009)	Filter paper	No	25%[a]	10	96	≈ 110[a] (glucose)
			54%[a]	10		≈ 90[a] (glucose)
Pristavka et al. (2000)	Salix caprea	SO_2 catalyzed steam-exploded	26.6%[a]	42	48	150-160 (glucose), 20 (xylose), 18 (disaccharides)

[a] The data are calculated according to the data from the reference other than directly cited.
[b] High solid loading was carried out by fed bath other than single batch.

Crystalline cellulose and sulfuric acid-pretreated corn stover were used as substrates to study high solid loading based saccharification (Olsen et al. 2011). Although the solid loading was up to 41% (w/v), the enzyme loading was very high (more than 70 FPU/g solid). The reaction was carried out for only 18 h, and the produced glucose concentration was close to 80 g/L. The continuously increasing trend of glucose concentration was small. It was reported that 103.3 g/L glucose was obtained from 96 h saccharification of steam-exploded corn stover (30%, w/v) with an enzyme loading of 20 FPU/g solid (Lu et al. 2010). Filter paper (high cellulose purity and low polymerization degree), was also used as substrate for high solid based saccharification with enzyme loading of 10 FPU/g solid (Kristensen et al. 2009). The produced glucose concentration was close to

110 g/L for 25% (w/v) solid loading and 90 g/L for 54% (w/v) solid loading, respectively. The produced sugar concentration in the above studies was lower than that from saccharification of alkali-pretreated sugarcane bagasse in our study. Although the produced sugar concentration from saccharification of alkali-pretreated wheat straw in our study was slightly lower than that from saccharification of steam-exploded corn stover (Lu et al. 2010) and filter paper (Kristensen et al. 2009), apparently higher enzyme loading was used in their studies. Besides, the produced sugar from willow saccharification (Pristavka et al. 2000) was 150 to 160 g/L, but the required enzyme loading was three times than that in our study.

Single batch other than fed batch was used in all above studies, where the viscosity of the reaction system was very high. This was why 54% (w/v) solid loading didn't bring higher glucose concentration than 25% (w/v) solid loading (Kristensen et al. 2009). Fed batch could alleviate the viscosity problem, and was proposed. The fed batch-based saccharification of corn stover was reported, and the produced glucose concentration was up to 175 g/L, which is the highest among all reports in Table 2 (Yang et al. 2010). It is confirmed that fed batch could decrease the viscosity of the reaction system and improve the hydrolytic efficiency. The enzymatic hydrolysis was initiated with 12% (w/v) solid loading and 20 FPU/g solid, and then 6% solid loading were fed at 12, 36, and 60 h. The final solid and enzyme loading was 30% (w/v) and 8 FPU/g solid, respectively. The product concentration is apparently higher, and the enzyme load is less compared to our study. However, the pretreatment in their study is very complicated. Before enzymatic hydrolysis, corn stover is pretreated by steam-explosion, NaOH, and H_2O_2 in turn. Complicated pretreatment inevitably incurs too high of a cost, hindering its industrial application. In contrast, wheat straw and sugarcane bagasse is only pretreated by NaOH in our study. So the application of high solid and low enzyme loading to produce high concentration sugar is relatively economical. Compared to wheat straw, sugarcane bagasse may a better lignocellulosic material for sugar production.

As shown in Table 1, more than 40% holocellulose (glucan and xylan) content remained in the enzyme-hydrolyzed materials. Further conversion of the remaining holocellulose to sugar could help increase the produced sugar concentration and decrease the materials cost, but it is very difficult for high solid and low enzyme loading based saccharification. Advances in this respect depend on developing efficient and economical pretreatment technology and decreasing the production cost of hydrolytic enzyme.

Besides, simple pretreatment requires a lower investment in equipment for a pilot or commercial plant, and simplicity of the unit procedure can also be helpful when designing a multifunctional batch reactor system (Gonzalez and Kafarov 2010).

ACKNOWLEDGEMENTS

This work was funded by the National Key Technology R&D Program (2011BAD22B01) and Knowledge Innovation Program of the Chinese Academy of Sciences (KSCX-YW-11-A3).

REFERENCES CITED

Casler, M. D., Mitchell, R., Richardson, J., and Zalesny, R. S. (2009). "Biofuels, bioenergy, and bioproducts from sustainable agricultural and forest crops," *BioEnergy Res*. 2(3), 77-78.

Ghose, T. K. (1987). "Measurements of cellulase activities," *Pure Appl. Chem*. 2(59), 257-268.

Gonzalez, A., and Kafarov, V. (2010). "Design of a multifunctional reactor for third generation biofuels production," *Chem. Eng. Trans.* 21(1297-1302.

Hodge, D. B., Karim, M. N., Schell, D. J., and McMillan, J. D. (2009). "Model-based fed-batch for high-solids enzymatic cellulose hydrolysis," *Appl. Biochem. Biotechnol*. 152(1), 88-107.

Jørgensen, H., Kristensen, J. B., and Felby, C. (2007). "Enzymatic conversion of lignocellulose into fermentable sugars: Challenges and opportunities," *Biofuels, Bioprod. Biorefining* 1(2), 119-134.

Kristensen, J. B., Felby, C., and Jorgensen, H. (2009). "Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose," *Biotechnol. Biofuels* 2.

Lu, Y. F., Wang, Y. H., Xu, G. Q., Chu, J., Zhuang, Y. P., and Zhang, S. L. (2010). "Influence of high solid concentration on enzymatic hydrolysis and fermentation of steam-exploded corn stover biomass," *Appl. Biochem. Biotechnol*. 160(2), 360-369.

Olsen, S. N., Lumby, E., McFarland, K., Borch, K., and Westh, P. (2011). "Kinetics of enzymatic high-solid hydrolysis of lignocellulosic biomass studied by calorimetry," *Appl. Biochem. Biotechnol*. 163(5), 626-635.

Pristavka, A., Kodituvakky, P. A., Kozlov, Y. P., Zacchi, G., Berezin, I. V., and Rabinovich, M. L. (2000). "High-solids enzymatic hydrolysis of steam-exploded willow without prior water washing," *Appl. Biochem. Microbiol*. 36(2), 101-108.

Yang, M. H., Li, W. L., Liu, B. B., Li, Q., Xing, J. M. (2010). "High-concentration sugars production from corn stover based on combined pretreatments and fed-batch process," *Bioresour. Technol*. 101(13), 4884-4888.

Zambare, V. P., Zambare, A. V., Muthukumarappan, K., Christopher, L. P. (2011). "Potantial of thermostable cellulases in the bioprocessing of awitchgrasss to ethanol," *BioResources* 6(2), 2004-2020.

Zhang, Y. H. P., Himmel, M. E., and Mielenz, J. R. (2006). "Outlook for cellulase improvement: Screening and selection strategies," *Biotechnol. Adv*. 24(5), 452-481.

Zhu, J. Y., Verrill, S. P., Liu, H., Herian, V. L., Pan, X., and Rockwood, D. L. (2011). "On polydispersity of plant biomass recalcitrance and its effects on pretreatment optimization for sugar production," *BioEnergy Res*. 4(3), 201-210.

Article submitted: September 18, 2011; Peer review completed: October 31, 2011; Revised version received: November 13, 2011; Accepted: November 14, 2011; Published: November 16, 2011.