Improved Ethanol Production Based on High Solids Fed-Batch Simultaneous Saccharification and Fermentation with Alkali-Pretreated Sugarcane Bagasse

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Alkali-pretreated sugarcane bagasse fiber was subjected to fed-batch simultaneous saccharification and fermentation (SSF) with a pre-hydrolysis process to increase the solids loading and produce a high concentration of ethanol. The hydrolysis medium and yeast feeding modes were investigated to determine suitable conditions for high sugar yield and ethanol production. Batch addition resulted in a cumulative substrate concentration of up to 36% (w/v) and enhanced ethanol concentrations, while ethanol conversion efficiency gradually declined. Enzymatic pre-hydrolysis and fermentation with fed-batch mode contributed to the SSF process. The highest ethanol concentration was 66.915 g/L with the conversion efficiency of 72.89%, which was achieved at 30% (w/v) solids content after 96 h of fermentation. Hydrolyzed medium and yeast were added in batch mode at 24 h of enzymatic hydrolysis and fermentation, respectively. Thus, combining the fed-batch mode with pre-hydrolysis SSF produced a high yield of ethanol.

Keywords: Simultaneous saccharification and fermentation; Pre-hydrolysis; Fed-batch process; Bioethanol; Sugarcane bagasse

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INTRODUCTION

Bioethanol production from lignocellulosic biomass requires the enzymatic hydrolysis of cellulose to release sugars that can be subsequently fermented by yeasts. For an economically viable ethanol production at the industrial level, the produced ethanol must reach at least 5% (v/v) (Varga *et al.* 2004). High substrate concentrations result in high sugar and ethanol concentrations, which require less energy and smaller equipment for a given process. Due to the volume reduction, the heating and cooling loads also are decreased. High solids saccharification with lower capital costs is a direct and convenient technique to obtain high concentration of sugars.

Simultaneous saccharification and fermentation (SSF) for ethanol production integrates substrate hydrolysis and fermentation in one reaction vessel, which allows for the released sugars from the hydrolysis to be rapidly consumed by the microbes, thereby eliminating end-product inhibition and reducing the investment cost. However, SSF is constrained by the typically suboptimal conditions for hydrolysis and fermentation, and limited by the available carbon sources for the microbes. These problems are reduced by adding a pre-hydrolysis step into SSF, which allows the enzymatic phase to operate at

optimal temperatures and yields more ethanol than other processes (Öhgren *et al.* 2006; Ko *et al.* 2009; Souza *et al.* 2012; Tan *et al.* 2013).

Simultaneous saccharification and fermentation at high solids loading can increase the bioethanol titer to facilitate downstream separation. In practical terms, high solids content is constrained by the rheological problems that include poor mass transfer, high-concentration inhibitors, and severe end-product inhibition (Olofsson *et al.* 2008; Kristensen *et al.* 2009; Zambare *et al.* 2011). Fed-batch SSF is an attractive strategy that potentially alleviates the difficulty of high-solids liquefaction for effective cellulose hydrolysis and fermentation.

Fed-batch fermentation has the advantage of increased maximum viable cell contents, which allows the product to accumulate to a high concentration; this method is widely used in industrial processes (Frison and Memmert 2002; Saarela *et al.* 2003). The critical process variables (*e.g.*, pH and temperature) can be maintained at specific levels through feedback controls (Gunther *et al.* 2007).

In this study, alkali-pretreated sugarcane bagasse (SCB) was used as a substrate for SSF. Delayed inoculation allowed increased substrate loading and the gradual feeding of hydrolyzed medium and yeast strategies. These variables were evaluated to enhance the final ethanol concentration.

EXPERIMENTAL

Materials and Pretreatment

SCB was obtained from Guangxi Fenghao Sugar Co., Ltd (Guangxi, China) and pre-milled into particles between 18 and 40 mesh size. The cellulase mixture, Cellic CTec2, was kindly provided by Novozymes A/S (Bagsaevrd, Denmark); it had an enzyme activity of approximately 200 FPU/mL, as measured by the IUPAC description (Ghose 1987).

Alkali pretreatment was performed with 0.5 M NaOH at a solid-to-liquid ratio of 1:20 in a round-bottom flask at 80 °C for 2 h with agitation. After pretreatment, the solid was separated by filtration and washed with tap water until it reached neutral pH. The obtained residues were dried in an oven at 65 °C and stored in a desiccator for the subsequent chemical analysis and the enzymatic hydrolysis experiments.

Microbial Strain and Inoculum

Saccharomyces cerevisiae Y-2034 was provided by L. J. Wickerham of the Northern Regional Research Laboratory of the United States Department of Agriculture (Washington DC, USA), was used as the inoculum for ethanol fermentation. It was stored at -80 °C in a 50% glycerol solution. After 24 h of activation, the yeast preparation, which contained nutrients, was added at a 10% (v/v) to a bioreactor.

Batch Fermentation with Pre-hydrolysis SSF

A series of batch enzymatic hydrolysis and fermentation experiments were conducted with solids loading of 12%, 18%, 24%, 30%, and 36% dry mass (DM) (w/v) at 50 °C and 150 rpm in 250-mL flasks, each containing 0.05 M sodium citrate buffer (pH 5.0). Four reactions were initiated with 18% (w/v) solids loading. For the processes with a loading higher than 18% (w/v), additional substrates containing 6%, 12%, or 18% (w/v) of solid were fed after 12 h of enzymatic hydrolysis. The enzyme loading was 10 FPU/g substrate, and it was dosed based on the final total amount of DM loaded into the system

at the beginning of the reaction. After 24 h of pre-hydrolysis, the systems were cooled to 37 °C and inoculated with yeast for the subsequent SSF process. Samples were withdrawn regularly at 6, 12, 24, 48, 72, and 96 h.

Fed-batch Fermentation with Pre-hydrolysis SSF

The fed-batch process was performed in two 500-mL Erlenmeyer reactors. In the first reactor, high solids fed-batch SSF with pre-hydrolysis was initiated with 18% (w/v) solid loading. Next, 6% (w/v) solid was fed at 12 h to increase the substrate loading to 24% (w/v). After 24 h of pre-hydrolysis, yeast cells were inoculated and enzymatic hydrolysis continued until the carbon source was exhausted. In the second reactor, a hydrolysis system containing a 36% (w/v) final solids loading was performed with an initial solids loading of 18% (w/v). Next, 12% (w/v) and 6% (w/v) solids were consecutively fed at 12 and 24 h, respectively. For the hydrolytic reactions, the enzyme loading was 10 FPU/g substrate, and it was dosed all at once at the beginning of the reaction based on the final total amount of DM loaded into the reactor. After the glucose in the first reactor was exhausted, the feeding of the hydrolyzed medium was started in two ways: batch at 24 h of fermentation, and fed-batch at 24 h (half medium) and 48 h (another half medium). Two comparable reactions were simultaneously carried out with different addition strategies of *S. cerevisiae*. For the conditions "A" and "B", the needed activated yeast cells were added in batch mode into the medium at the beginning of fermentation; for "C" and "D", the yeast cells were consecutively fed at the fermentation times of 0 h with 5% (v/v) and 24 h with the other 5% (v/v), respectively (Table 1). In these reactions, the hydrolyzed medium and yeast were dosed to maintain a constant ratio of yeast to substrate in the reaction system (Table 1). At each desired time, sample solutions were taken out for sugar and ethanol analysis.

Table 1. Substrate and Yeast Feeding Scheme for the Different Reactions

Run No.	Hydrolyzed medium Addition Mode		Yeast Addition Mode	
	Fermentation Time (h)	Feeding Amount (mL)	Hydrolysis Time (h)	Feeding Amount (%)
A	24	100 (contained 36 g solids)	24	10
	48	—	48	—
B	24	50 (contained 18 g solids)	24	10
	48	50 (contained 18 g solids)	48	—
C	24	100 (contained 36 g solids)	24	5
	48	—	48	5
D	24	50 (contained 18 g solids)	24	5
	48	50 (contained 18 g solids)	48	5

Analysis Methods

The components of the SCB before and after the pretreatment were determined according to a standardized method (Sluiter *et al.* 2008). Sugars (glucose, xylose, cellobiose, and arabinose) and fermentation acids (acetic acid, formic acid, and glucuronic acid) were analyzed by high-performance liquid chromatography (HPLC, Waters model 2695, Wilford, USA) using a Shodex sugar SH-1011 column coupled with a refractive index detector (Waters 2414 RI) at 50 °C. The mobile phase was 5 mmol/L of H_2SO_4 at a flow rate of 0.5 mL/min. Ethanol was analyzed using gas chromatography (model 6820,

Agilent, Santa Clara, USA) equipped with a flame ionization detector (FID) and a fused-silica capillary column (DB-FFAP, 30 m × 0.25 mm × 0.25 µm).

RESULTS AND DISCUSSION

Composition Changes after Pretreatment

High ethanol production requires a high cellulose concentration. Increased cellulose content is mostly affected by the removal of non-cellulose components *via* pretreatment. Lignin is a protective physical barrier to enzyme attack. Under alkaline conditions, lignin is almost completely removed from the lignocellulosic biomass (Chaudhary *et al.* 2012). To facilitate the rapid and efficient hydrolysis of carbohydrates, alkaline delignification pretreatment was used in this study.

Raw SCB was mainly composed of glucan (38.61%), xylan (23.63%), and lignin (21.21%). Alkali pretreatment removed the majority of lignin. The Klason lignin content was reduced by approximately 62.4%; only 7.97% remained in the pretreated residues. Simultaneously, the glucan and xylan contents were increased to 60.11% and 25.52%, respectively, and the cellulose content of the SCB after treatment was increased proportionally by 55.69%. These changes facilitated the subsequent enzymatic hydrolysis operating at high substrate loading (Zhang *et al.* 2013).

Batch Fermentation-Based Pre-hydrolysis SSF

Separate hydrolysis and fermentation (SHF) and SSF are two methods generally used for bioethanol production. Simultaneous saccharification and fermentation is favored over SHF for its ability to promptly convert the glucose released by cellulose into bioethanol, thus minimizing end-product inhibition and cellobiose accumulation (Zaldivar *et al.* 2001). In the SHF process, enzymatic hydrolysis can be operated at a higher temperature than SSF, and consequently the process results in enhanced enzyme activities. The pre-hydrolysis SSF process allows for the enzymatic phase to be operated under optimal conditions prior to fermentation. It integrates the advantages of both SSF and SHF, which could increase ethanol productivity if suitable conditions are selected.

In our previous experiments, the optimal pre-hydrolysis time (24 h) was established for SSF (Liu *et al.* 2015). Substrate concentration also affects the final ethanol yield. Enzymatic hydrolysis and ethanol production were investigated with different solid loading levels (12 to 36% (DM) (w/v)) during SSF with a 24 h delayed inoculation.

Figure 1 shows the changes in the concentration of sugars and the ethanol yield after 96 h of fermentation. Glucose was almost exhausted before 48 h in all of the experiments, and the ethanol concentration increased rapidly after the first 24 h of fermentation. The ethanol production rate was consistent with the glucose consumption. A considerable quantity of xylose was also detected. Substrate loading from 12 to 36% (w/v) DM caused 117.13% (from 18.692 to 38.441 g/L) and 33.97% (from 25.752 to 55.916 g/L) increases in ethanol and xylose, respectively. Some unconverted cellobiose was retained in the stillage during glucose consumption. As cellobiose accumulated and the substrate increased, glucose conversion decreased.

Furthermore, the ethanol conversion efficiency decreased as the substrate increased (Fig. 2A). At 36% (w/v) solid loading, the ethanol conversion efficiency was decreased by approximately 27.62% (from 70.13 to 50.76%) compared with the 12% solids content. With loading higher than 24%, the ethanol conversion efficiency was considerably reduced.

This result was likely caused by the high viscosity in high substrate content slurries, which reduced the hydrolysis and fermentation efficiencies. Moreover, high solids loading increased the concentrations of inhibitors such as organic acids (Fig. 2B) and other accumulated by-products (Jorgensen *et al.* 2007). The produced acids may change the osmotic pressure of the yeast cells, and the sensitivity of the yeast to fermentation by-products (Fig. 2B) may have been affected as the solids content increased.

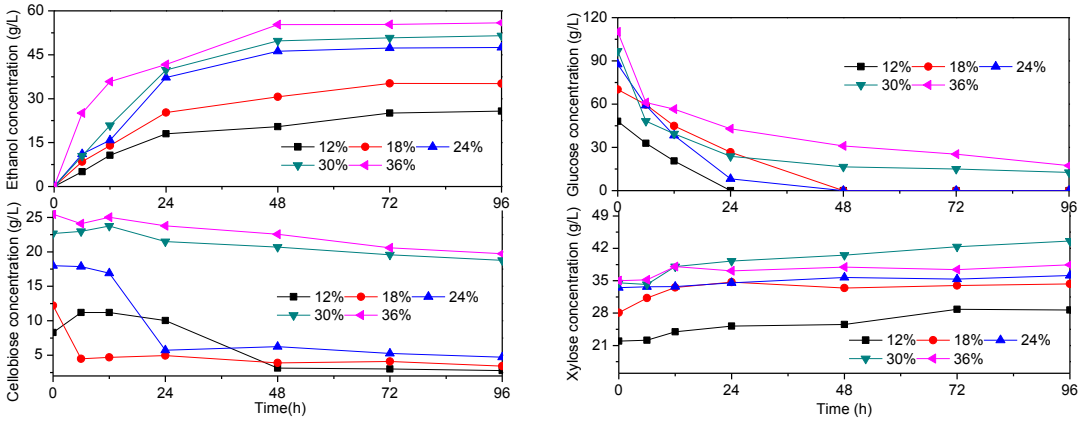

Fig. 1. Sugar and ethanol concentration in batch fermentation-based pre-hydrolysis SSF with different substrate loadings (indicated in the legend)

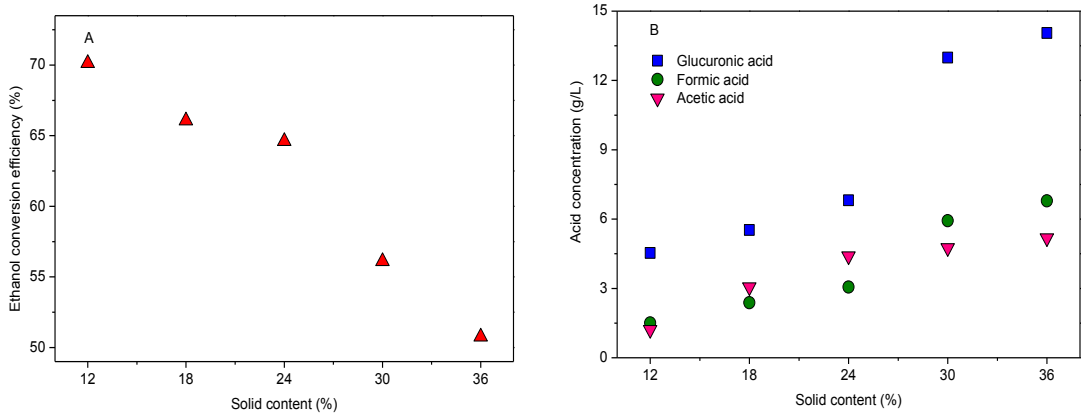

Fig. 2. (A) Ethanol conversion efficiency and (B) acid concentration in batch-based pre-hydrolysis SSF after 96 h of fermentation with different substrate loadings

Fed-Batch Pre-hydrolysis SSF

To minimize the negative effects caused by the high-solids system, an improved culture method for high ethanol production with *S. cerevisiae*, fed-batch SSF, was carried out to determine the suitable conditions. The pretreated substrates, hydrolyzed medium, and yeast cells were added during the enzymatic pre-hydrolysis and fermentation stages according to the scheme in Table 1.

Figures 3 and 4 compare the sugar concentrations and ethanol production from the fed-batch based pre-hydrolysis SSF process, with 30% final solids loading under four different feeding conditions. The fed-batch SSF approach improved the sugar and ethanol concentrations for the different conditions compared with the SSF carried out with 30%

solids loading in batch fermentation, in which the ethanol concentration was 51.503 g/L with a conversion efficiency of 56.10% (Figs. 1, 2). Furthermore, the ethanol production achieved better results when all the yeast was added at the beginning of the fermentation, *i.e.*, the A and B conditions, compared with conditions C and D. Comparisons between the four conditions indicated that the feeding time and feeding amount affected the hydrolysis and fermentation system performance.

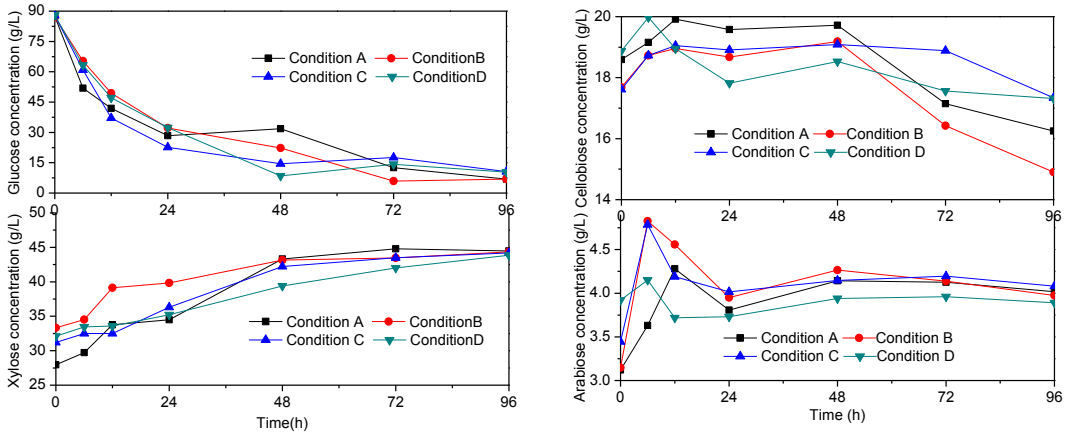

Fig. 3. Sugars and ethanol concentrations in pre-hydrolysis SSF with different fed-batch modes

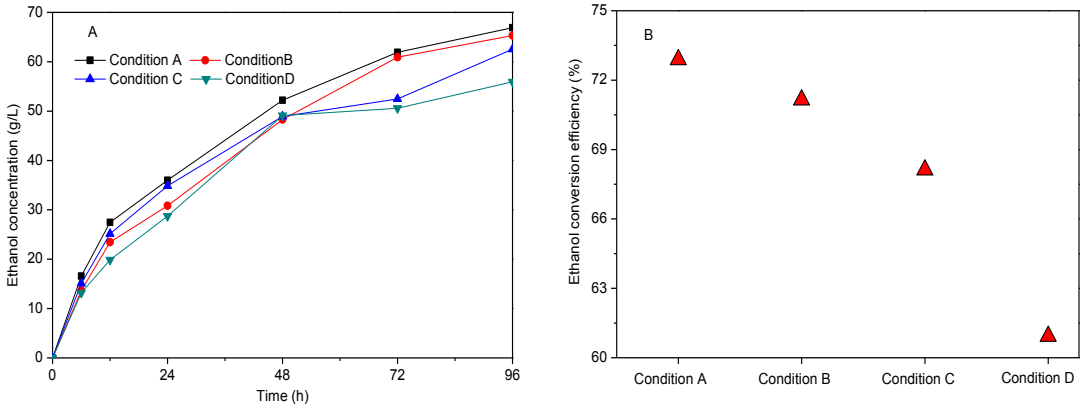

Fig. 4. (A) Ethanol concentration and (B) conversion efficiency after 96 h of fermentation in different fed-batch mode

The best result was achieved with condition A, when both the hydrolyzed medium and yeast were added in batch mode at 24 h of enzymatic hydrolysis and fermentation, respectively (Fig. 4). The ethanol concentration and conversion efficiency reached 66.918 g/L and 72.89%, respectively, after 96 h of fermentation with condition A. In contrast, for condition D, the ethanol concentration and conversion efficiency were only 55.934 g/L and 60.93%, respectively, when the hydrolyzed medium and yeast were added twice and separately at different feeding points. In sum, condition A produced higher sugar and ethanol contents than the other operating modes. The result was higher than the values reported by other authors. Lu *et al.* (2010) obtained 49.5 g/L ethanol with 30% solids loading. Gao *et al.* (2014) used 25% solids loading only achieved 36.25 g/L ethanol after 96 h fermentation.

There are several plausible reasons for this result. Batch feeding of the hydrolyzed medium supplied sufficient carbon for the yeast cells in a timely manner, which was added at the beginning of the fermentation and had already adapted to the environment, and the yeast stability increased the ethanol production rate during the initial stage of the fermentation. Moreover, the shorter feeding intervals enabled the enzyme to keep high-activity in both reactors simultaneously, which led to the further hydrolysis of substrates and quickly decreased the viscosity of the system. The relatively sufficient liquefaction and fermentation of the substrates also reduced the fermentation acid by-products (Fig. 5). Based on these results, it was concluded that the interplay between the substrate and yeast-feeding mode affected the entire reaction process.

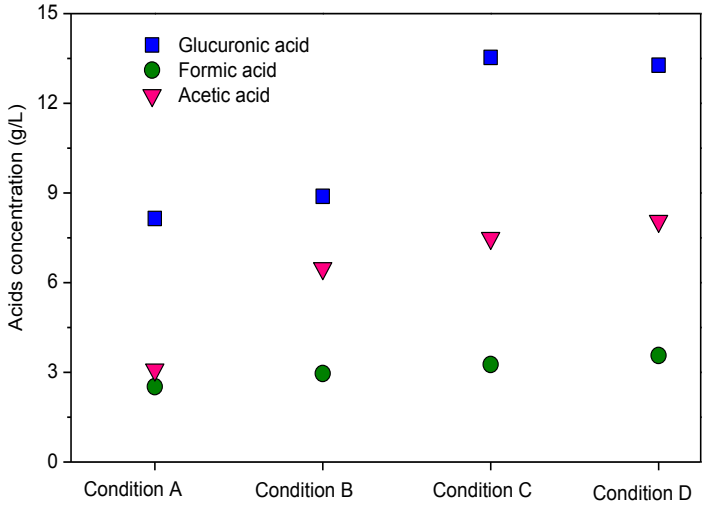

Fig. 5. Acid concentration after 96 h of fermentation in different fed-batch modes

CONCLUSIONS

1. Simultaneous saccharification and fermentation with pre-hydrolysis eliminated the typical problems of SSF. A stepwise substrate addition mode permitted high cumulative substrates loading (36%, w/v) and produced relatively high levels of ethanol (55.916 g/L). However, the conversion of glucose to ethanol was inhibited as the mixing difficulty increased due to heat transfer problems caused by the high-solids system.
2. Enzymatic pre-hydrolysis and fermentation with the fed-batch feeding strategy improved the ethanol productivity and had a positive effect on the overall ethanol yield. The highest ethanol concentration of 66.915 g/L with a conversion efficiency of 72.89% was reached at a solid loading of 30% (w/v) after 96 h of fermentation, when the hydrolyzed medium and yeast were added in batch mode after 24 h of enzymatic hydrolysis and fermentation, respectively.

3. Fed-batch mode with pre-hydrolysis SSF process can effectively increase solids loading, obtain high-concentrations of ethanol and high cellulose conversion efficiencies and finally reduce the production cost. The process presented here might be a plausible solution for economic ethanol production systems.

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REFERENCES CITED

Chaudhary, G., Singh, L. K., and Ghosh, S. (2012). "Alkaline pretreatment methods followed by acid hydrolysis of *Saccharum spontaneum* for bioethanol production," *Bioresource Technol.* 124, 111-118. DOI: 10.1016/j.biortech.2012.08.067

Frison, A., and Memmert, K. (2002). "Fed-batch process development for monoclonal antibody production with cellferm-pro," *Gen. Eng. News* 22, 66-67.

Gao, Y. S., Xu, J. L., Yuan, Z. H., Zhang, Y., Liang, C. Y., and Liu, Y. Y. (2014). "Ethanol production from high solids loading of alkali-pretreated sugarcane bagasse with SSF process," *BioResources* 9(2), 3466-3479. DOI: 10.15376/biores.9.2.3466-3479

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

Gunther, J. C., Seborg, D. E., and Baclaski, J. (2007). "Fault detection and diagnosis in industrial fed-batch cell culture process," *Biotechnol. Progr.* 23(4), 851-857. DOI: 10.1021/bp070063m

Jorgensen, H., Vibe-Pedersen, J., Larsen, J., and Fellby, C. (2007). "Liquefaction of lignocelluloses at high-solids concentrations," *Biotechnol. Bioeng*. 96(5), 862-870. DOI: 10.1002/bit.21115

Ko, J. K., Bak, J. S., Jung, M. W., Lee, H. J., Choi, I. G., Kim, T. H., and Kim, K. H. (2009). "Ethanol production from rice straw using optimized aqueous-ammonia soaking pretreatment and simultaneous saccharification and fermentation processes," *Bioresource Technol.* 100, 4374-4380. DOI: 10.1016/j.biortech.2009.04.026

Kristensen, J. B., Felby, C., and Jorgensen, H. (2009). "Yield determining factors in high-solids enzymatic hydrolysis of lignocellulose," *Biotechnol. Biofuels* 2, 11. DOI: 10.1186/1754-6834-2-11

Liu, Y. Y., Xu, J. L., Zhang, Y., Yuan, Z. H., He, M. C., Liang, C. Y., Zhuang, X. S., and Xie, J. (2015). "Sequential bioethanol and biogas production from sugarcane bagasse based on high solids fed-batch SSF," *Energy* 90, 1199-1205. DOI: 10.1016/j.energy.2015.06.066

Lu, Y. F., Wang, H. Y., 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. Biotech.* 160(2), 360-369. DOI: 10.1007/s12010-008-8306-0

Öhgren, K., Rudolf, A., Galbe, M., and Zacchi G. (2006). "Fuel ethanol production from steam-pretreated corn stover using SSF at higher dry matter content," *Biomass Bioenerg.* 30(10), 863-869. DOI: 10.1016/j.biombioe.2006.02.002

Olofsson, K., Bertilsson, M., and Lidén, G. (2008). "A short review on SSF - An interesting process option for ethanol production from lignocellulosic feedstocks," *Biotechnol. Biofuels.* 1(1), 7. DOI: 10.1186/1754-6834-1-7

Saarela, U., Leiviska, K., and Juuso, E. (2003). *Modelling of a Fed-Batch Fermentation Process*, Technical Report A No. 21, University of Oulu, Finland.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., and Crocker, D. (2008). *Determination of Structural Carbohydrates and Lignin in Biomass: Laboratory Analytical Procedure*, National Renewable Energy Laboratory, Golden, CO, USA, (http://www.nrel.gov/biomass/pdfs/42618.pdf).

Souza, C. J., Costa, D. A., Rodrigues, M. Q., dos Santos, A. F., Lopes, M. R., Abrantes, A. B., dos Santos, C. P., Silverira, W. B., Passos, F. M., and Fietto, L. G. (2012). "The influence of presaccharification, fermentation temperature and yeast strain on ethanol production from sugarcane bagasse," *Bioresource Technol.* 109, 63-69. DOI: 10.1016/j.biortech.2012.01.024

Tan, L., Tang, Y. Q., Nishimura, H., Takei, S., Morimura, S., and Kida, K. (2013). "Efficient production of bioethanol from corn stover by pretreatment with a combination of sulfuric acid and sodium hydroxide," *Prep. Biochem. Biotechnol.* 43, 682-695. DOI: 10.1080/10826068.2013.773338

Varga, E., Klinke, H. B., Reczey, K., and Thomsen, A. B. (2004). "High solid simultaneous saccharification and fermentation of wet oxidized corn stover to ethanol," *Biotechnol. Bioeng.* 88(5), 567-574.

Zaldivar, J., Nielsen, J., and Olsson, L. (2001). "Fuel ethanol production from lignocellulose: A challenge for metabolic engineering and process integration," *Appl. Microbiol. Biotechnol.* 56(1), 17-34. DOI: 10.1007/s002530100624

Zhang, Y., Xu, J. L., Yuan, Z. H., Liu, Y. Y., and He, M. C. (2013). "Comparison of different pretreatment methods on sugarcane bagasse and fractal-like kinetics of enzymatic hydrolysis," *Adv. New Renew. Energy* 1(2), 166-169. DOI: 10.3969/j.issn.2095-560X.2013.02.007

Zambare, V. P., Zambare, A. V., Muthukumarappan, K., and Christopher, L. P. (2011). "Potential of thermostable cellulases in the bioprocessing of switchgrass to ethanol," *Bioresources* 6(2), 2004-2020. DOI: 10.15376/biores.6.2.2004-2021

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