OPTIMIZING SULFITE PRETREATMENT FOR SACCHARIFICATION OF WHEAT STRAW USING ORTHOGONAL DESIGN

Jiayi Yang,[a] Gaosheng Wang,[a,b*] Lindong Qi,[a] and Jie Xu [a]

An orthogonal designed experiment was used to investigate the effects of sulfite pretreatment on the components separation and saccharification of wheat straw. The process involved sulfite pretreatment of wheat straw under acidic conditions followed by mechanical size reduction using a high consistency refiner. Reaction temperature, retention time, and charges of sodium bisulfite and sulphuric acid were considered as key factors. The results showed the four factors had impact on saccharification of wheat straw. Raising the temperature, increasing the charge of sodium bisulfite or sulphuric acid, or extending the retention time would improve the dissolution of pentosan, lignin, and saccharification efficiency, while causing further conversion of pentose. The separation of lignin and pentosan from wheat straw was the main cause of improvements in saccharification. With an enzyme loading of 5 FPU cellulase plus 4 CBU β-glucosidase per gram of o.d. substrate, a glucose yield 72.45% was achieved using the substrate pretreated under the conditions of temperature 180 ℃, sodium bisulfite charge 3%, sulfuric acid charge 1.48%, and retention time 20 min.

Keywords: Sulfite; SPORL; Pretreatment; Wheat straw; Saccharification; Optimization

*Contact information: a: Tianjin Key Lab of Pulp and Paper, Tianjin University of Science and Technology, Tianjin, 300457, P.R. China; b: State Key Lab of Pulp and Paper, South China University of Technology, Guangzhou, 510640, P.R. China; * Corresponding author: gswang@tust.edu.cn*

INTRODUCTION

Lignocellulose, the most abundantly renewable biomass produced by photosynthesis, has a yearly supply of approximately 200 billion metric tons worldwide available for conversion into fuel and chemicals (Ragauskas et al. 2006; Zhang et al. 2006b). A transition from a fossil fuel-based economy to a renewable carbohydrate economy will inevitably take place in the foreseeable future because of the depletion of fossil fuel reserves and the accumulation of greenhouse gases (Caldeira et al. 2003; Demain et al. 2005; Farrell et al. 2006; Ragauskas et al. 2006; Wingren et al. 2003).

Pretreatment is one of the key elements in bioconversion of lignocellulosic material to bio-ethanol, in which the efficient enzymatic hydrolysis of biomass is required to overcome the physical and chemical barriers that inhibit the accessibility of enzyme to the substrate (Saddler 1993). Among the known chemical barriers are lignin, hemicellulose, and the acetyl group (Chang and Holtzapple, 2000; Grohmann et al. 1989; Kong et al. 1992). Physical factors, such as crystallinity (Caufield and Moore 1974; Cowling and Kirk 1976; Fan et al. 1980; Polcin and Bezuch 1977; Sasakiet al. 1979; Schwald et al. 1988), surface area (Burns et al. 1989; Lee et al. 1995), and degree of polymerization (Puri 1984), are also known to affect enzymatic hydrolysis. Among these factors, lignin and hemicellulose were considered as the major impeding factors.

Separation of the biomass into the three main biomass constituents is a concept being developed as a means to promote the overall biomass utilization. Hemicellulose, when separated from the biomass, may be used in a broad range of applications, including chemicals, fuel, and food. The lignin separated can also be used in multiple ways, such as a fuel, noting that the energy content of lignin is 26.3 MJ/OD kg (Saddler, 1993). Various pretreatment options have been applied to fractionate cellulose, hemicellulose, and lignin from lignocellulosic materials. Chemical hydrolysis of biomass is relatively efficient and economically feasible. However, it generates fermentation inhibitors (Lether 2003). On the other hand, enzymatic hydrolysis, despite its relatively slow rate, is a biocompatible and environmentally friendly option. All the above options have achieved some level of success, but some critical issues still need to be solved: (1) Most existing pretreatment processes have low cellulose conversion, except for the organosolv process. (2) Slow rates of enzymatic hydrolysis resulting from limited removal of recalcitrant components from the material adversely affects process efficiency (Philippidis and Smith 1995) and therefore the economics of cellulosic ethanol production (Hinman et al. 1992). (3) Most of the existing pretreatment technologies have poor scalability due to lack of commercially proven capital equipment.

The SPORL process, which is also known as Sulfite Pretreatment to Overcome Recalcitrance of Lignocellulose, was recently developed and is new to the biomass research community (Zhu et al. 2009, 2010; Wang et al. 2009). The development of the SPORL process is based on the following fundamental understandings of sulfite pulping and considerations: (1) a considerable amount of hemicellulose degradation and removal takes place during sulfite pulping, as evidenced by the predominant pentosan content in pulping spent liquor; (2) the degrees of polymerization of xylan and cellulose are reduced; (3) sulfonation of lignin increases the hydrophilicity of lignin, which may promote the aqueous enzyme process; and (4) the degrees of dissolution of hemicellulose, degradation of cellulose, and sulfonation and condensation of lignin are increased as reaction time and temperature increase, and as pH decreases.

The previous studies on SPORL process were focus on woody biomass, including softwood and hardwood (Zhu et al. 2009; Wang et al. 2009). This paper applied SPORL for efficient conversion of wheat straw through enzymatic hydrolysis. Wheat straw is an important source of lignocellulosic biomass for a wide variety of applications and large output, and has a broad prospect for industrialization. In the present work a wide range of reaction and operating conditions were explored to seek the optimum range of the process parameters that allow satisfactory pretreatment and components separation of wheat straw.

EXPERIMENTAL

Material and Reagents

Wheat straw was obtained from Jinghai, Tianjin, China. The initial compositions of wheat straw were shown in Table 1. Commercial cellulase and β-glucosidase were provided by Novozyme Company. Tetracycline hydrochloride was purchased from Solarbio Company. Sodium bisulfite, sulphuric acid, and sodium hydroxide were analytically pure.

Table 1. Composition Analysis of Wheat Straw (%)

Compositions	ash	lignin	Acid Soluble lignin	Acid in-soluble lignin	Benzene-alcohol extractive	glucan	pentosan
Content (%)	7.93	16.67	1.88	14.79	4.30	36.74	23.56

Pretreatment Operation

The pretreatment liquor was prepared by mixing sodium bisulfite with sulphuric acid. Wheat straws mixed with different pretreatment liquor were placed into eight sealed stainless vessels. The vessels were heated via hot air bath and rotating at a speed of 2 rpm during pretreatment. Wheat straw was directly subjected to pretreatment using sodium bisulfite with or without sulfuric acid prior to size reduction. The ratio of pretreatment liquor to wheat straw (o.d.) was 4:1(v/w). The orthogonal test of L_{16} (4^4) was adopted to investigate the significance of the factor and optimize the pretreatment. A mean value of for each condition was used in the calculation. The factors and levels of the orthogonal experiment are shown in Table 2.

Table 2. L_{16} (4^4)-Orthogonal Design for Sulfite Pretreatment*

Level	Temp. °C	Bisulfite Charge %	Sulfuric acid charge %	Retention time min
1	160	0.0	0.0	10
2	170	1.5	0.74	20
3	180	3.0	1.48	30
4	190	4.5	2.22	40
* The solid-to-liquor ratio was 1:4 for every trial.				

At the end of the process, the vessels were cool down to lower than 100 °C using water, then the pretreated sample was directly transferred to a disk refiner for size reduction. The weight loss for each sample was determined from the measured wet weight and moisture content of the substrate. Size reduction was carried out with disk gap of 0.2 mm. The refined sample was collected after refining and stored for further determination and enzymatic hydrolysis.

Enzymatic Hydrolysis

Enzymatic hydrolysis of the substrates was carried out at a substrate consistency of 2% (w/v) in 50 mL sodium acetate buffer (pH 4.8) at 50 °C using a shaking incubator at 200 rpm. A manufacturer-specified activity of 70 FPU/g of cellulase and 250 CBU/g of β-glucosidase was used to calculate enzyme loading. A mixture of cellulase with an activity loading of 5 FPU/g substrate and β-glucosidase with an activity loading of 4 CBU/g substrate was used for enzymatic hydrolysis. The recommended enzyme load from Novozyme was applied in the tests. Excessive β-glucosidase was used to prevent cellobiose accumulation (Emmel et al. 2003). Hydrolysates were taken periodically at appropriate sampling times (1, 3, 6, 12, 24, 48, and 72 h) for determination of glucose content. Totally released glucose after 72 h of hydrolysis was used to estimate the enzymatic digestibility.

Analytical Method

The contents of the main components of wheat straw are described in Table 1. Glucose (or glucan) contents of material, substrate, and hydrolysates were directively determined by a commercial glucose analyzer (SBA 40-D glucose analyzer, Jinan, Shandong, China). Relative error was about 1% based on manufacturer specifications. The Douglas colorimetric method was adopted to determine the contents of pentosan in spent liquor, substrate, and material (Yu et al. 2007). Lignin contents determination in spent liquor and solid samples were analyzed following the procedure of GB/T standard NO. 2677.8-1994 and employed an ultraviolet spectrophotometer (756PC, Shanghai Optical Spectrum Instrument Ltd. China). Wavelength accuracy and photometric accuracy of the ultraviolet spectrophotometer were about 0.5 nm and ±0.5 %T, respectively. Each sample was analyzed in duplicate, and the average of duplicate runs was used in reporting.

Calculation

The following five equations indicate the parameters used to evaluate the pretreatment, which are cellulose conversion (%CC), glucose yield (%GY), digestion ratio of pentosan (%DRP), retention ratio pentosan (%RRP), and delignification ratio of lignin (%DRL). The terms, “%DRP” and” %RRP”, were used to depict the change of pentosan in pretreatment liquor and substrate during the process respectively.

$$\%\mathrm{CC} = \frac{C_1(\mathrm{g}/L) \times 0.1L \times 0.9}{2g \times Content_1(\%)} \times 100\% \qquad (1)$$

$$\%\mathrm{GY} = \frac{C_1(\mathrm{g}/L) \times 0.1L \times 0.9}{[2g/Y_1(\%)] \times Content_2(\%)} \times 100\% \qquad (2)$$

$$\%\mathrm{DRP} = \frac{C_2(\mathrm{g}/L) \times 0.4L}{Content_3(\%) \times 100g} \times 100\% \qquad (3)$$

$$\%\mathrm{RRP} = \frac{W_1(g) \times Content_4(\%)}{[W_1(g)/Y_1(\%)] \times Content_3(\%)} \times 100\% \qquad (4)$$

$$\%\mathrm{DRL} = \frac{C_3(\mathrm{g}/L) \times 0.4L}{Content_5(\%) \times 100g} \times 100\% \qquad (5)$$

In these equations C_1 is the glucose concentration in enzymatic hydrolysates; C_2 is the pentosan concentration in liquor; C_3 is the lignin concentration in the liquor; $Content_1$ is the glucose content in substrate; $Content_2$ is the glucose content in material; $Content_3$ is the pentosan content in material; $Content_4$ is the pentosan content in substrate; $Content_5$ is the lignin content in material; W_1 is the weight of substrate; and Y is the substrate yield.

RESULTS AND DISCUSSION

A $L_{16}(4^4)$ orthogonal experiment was adopted to investigate the effects of the four factors (temperature, charges of bisulfite and sulfuric acid, and retention time) on the pretreatment of wheat straw. The indexes considered were cellulose conversion, glucose yield, pentosan yield, retention ratio of pentosan, and delignification ratio of lignin.

Effects of Bisulfite Charge on Pretreatment of Wheat Straw

The effects of bisulfite charge on pretreatment of wheat straw are shown in Figs. 1 to 3. In Fig.1, the %DRP and %RRP are plotted against the bisulfite charge. As shown by the fitted curves, %DRP increased with the increasing dosage of bisulfite, while the %RRP followed an opposite trend. The regression coefficients indicate that the experimental values agreed well with the fitting curves, especially the digestion ratio of pentosan. The degradation of hemicellulose increased from 31% to 53% when the charge ranged from 0% to 4.5%. The pH value of the liquor decreased with the addition of more sodium bisulfite in the process, while the degradation of hemicellulose should be aggravated in the weakly acidic liquor. More hemicellulose degraded from the wheat straw caused opposing currents, resulting in an increased digestion ratio and the decreasing of retention ratio.

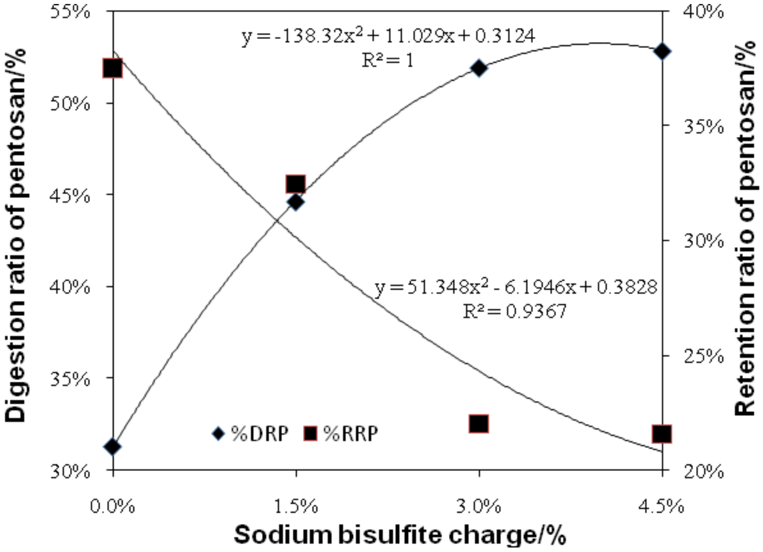

Fig. 1. Effect of sodium bisulfite charge on yield and retention ratio of pentosan

The %DRL change with bisulfite dosage is shown in Fig. 2, along with the curve representing the best fit. The lignosulfonate, the outcome of a sulfonation reaction between sulfite and lignin, was generated in increasing amounts with increasing dosage of sodium bisulfite. The lignosulfonate can weaken the hydrophobic interaction between the substrate and enzymes, and make the lignin carbohydrate complex (LCC) more hydrophilic. The increasing of cellulose conversion and glucose yield can be seen from Fig. 3. The cellulose conversion and glucose yield at the 4.5% dosage was not much higher than at the 3.0% dosage. It is obvious that both glucose yield and cellulose conversion followed the trend of the fitted curve, which means that the optimized value from the nonlinear regression was in accordance with exact experimental value. Comparing the dosages of 0% and 4.5%, one can find that the enzymatic digestibility of a sulfite-treated substrate was significantly improved by applying more sodium bisulfite. The partial delignification and lignin sulfonation all contributed to the significantly improved digestibility of the substrate. The separation of lignin and hemicellulose from the raw material favored the enzyme adsorption to the cellulose, which is absolutely important to the enzymatic hydrolysis. Anyway, the increasing of sodium bisulfite application depressed the degradation and dissolution of hemicellulose and lignin, while overcoming the recalcitrance of the substrate. This enhanced the accessibility in the enzymatic hydrolysis and raised the final saccharification efficiency.

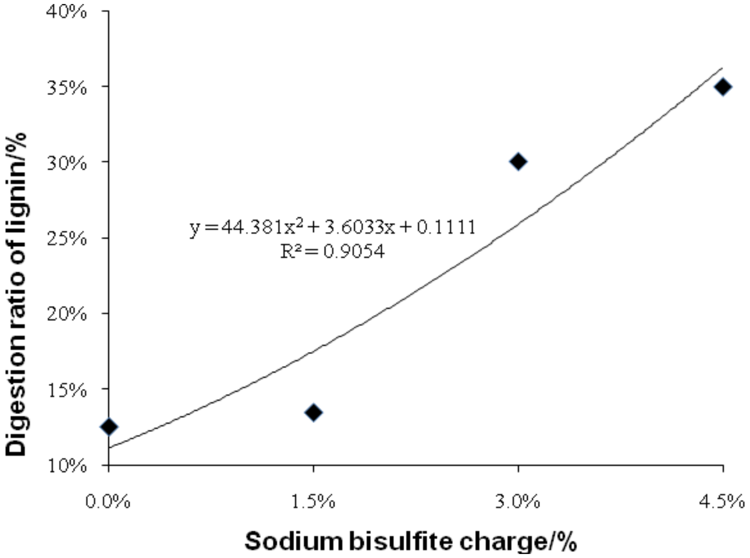

Fig. 2. Effect of sodium bisulfite charge on digestion ratio of lignin

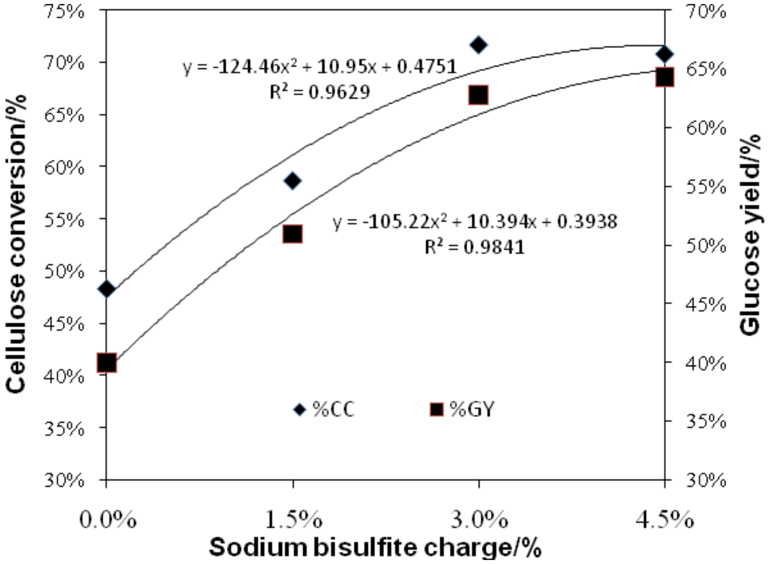

Fig. 3. Effect of sodium bisulfite charge on cellulose conversion and glucose yield

Effects of Sulphuric Acid Charge on Pretreatment of Wheat Straw

The effects of sulfuric acid charge on the components separation and saccharification are shown in Figs. 4, 5, and 6. The results indicate that sulfuric acid charge had an important impact on the digestion ratios of pentosan and lignin. Comparing the fitted curves in Figs. 4 and 5, in which the acid dosage changed from 0% to 2.2%, the dissolution of hemicellulose and lignin were improved when using more sulfuric acid, while causing the retention ratio of pentosan to have a downward tendency. Higher dosage of sulfuric acid in the pretreatment resulted in an acidic condition for the degradation of hemicellulose and lignin. As sulfuric acid charge increasing, the separation of hemicellulose and lignin from lignocellulose increased, improving the cellulose conversion and glucose yield.

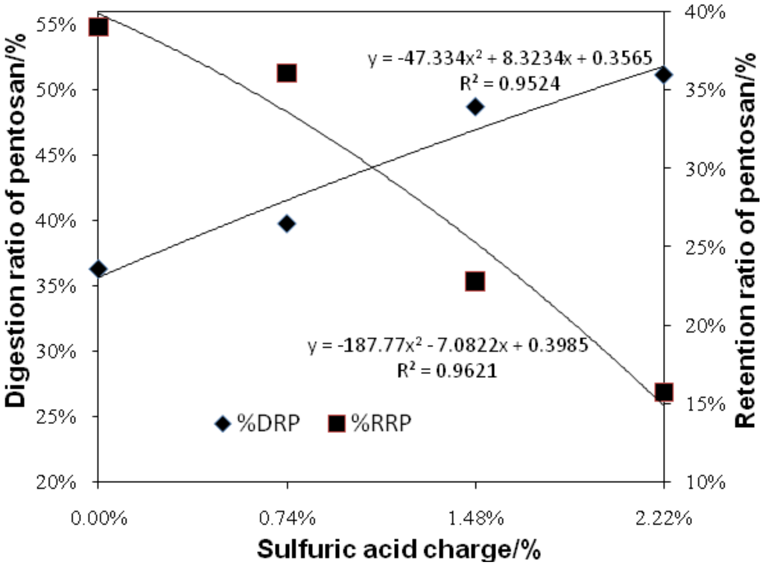

Fig. 4. Effect of sulfuric acid charge on yield and retention rate of pentosan

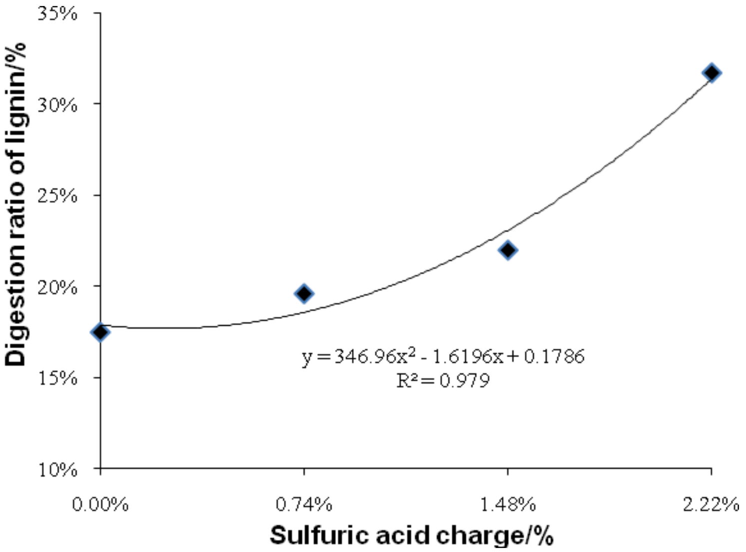

Fig. 5. Effect of sulfuric charge on dissolution of lignin

However, that dosage of sulfuric acid at a relative higher temperature would accelerate the conversion of pentosan, the condensation of lignin, and also the degradation of cellulose. In the pretreatment at high temperature with lower pH value, hemicellulose and cellulose tended to depolymerize and was converted into fermentation inhibitors. As was shown in Fig. 4, pentosan yield (%DRP plus %RRP) was about 66%, which meant that over 30% of the pentosan was converted into other chemicals at the acid charge of 2.2%. The low cellulose conversion after pretreatment with lower sulfuric acid charge can be attributed to the remaining hemicellulose, which blocked the enzymatic catalysis during the hydrolysis of the cellulose. Furthermore, the residuals would cause the ineffective adsorption of cellulase to hemicellulose. The condensed lignin also had chemical/physical structures affecting the enzymatic hydrolysis more than the absolute amount of lignin.

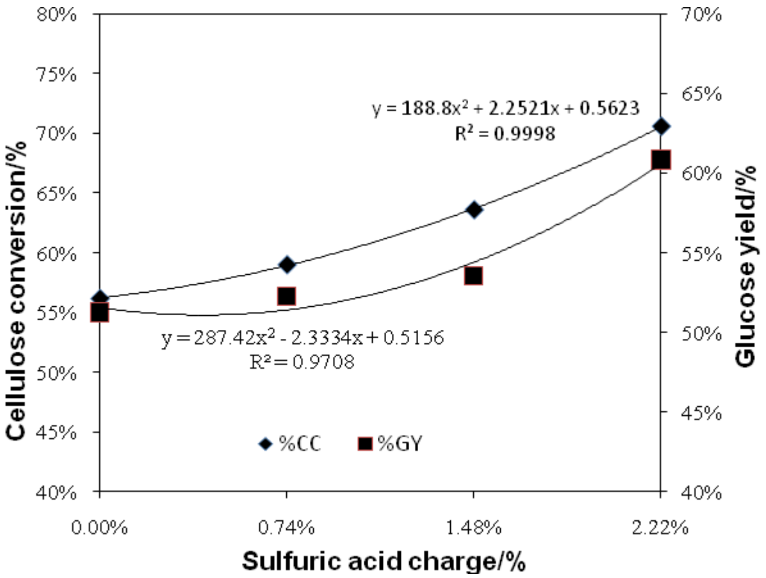

Fig. 6. Effect of sulfuric acid charge on cellulose conversion and glucose yield

Effects of Reaction Temperature on Pretreatment of Wheat Straw

Figures 7, 8, and 9 show how the reaction temperature influenced the sulfite pretreatment. In Fig.7, the %DRP and %RRP are plotted against the temperature of the trial. As the fitting curves show, the dissolved hemicellulose in the spent liquor caused a convex trend in the whole temperature range; the %DRP was 22% at 190 °C.

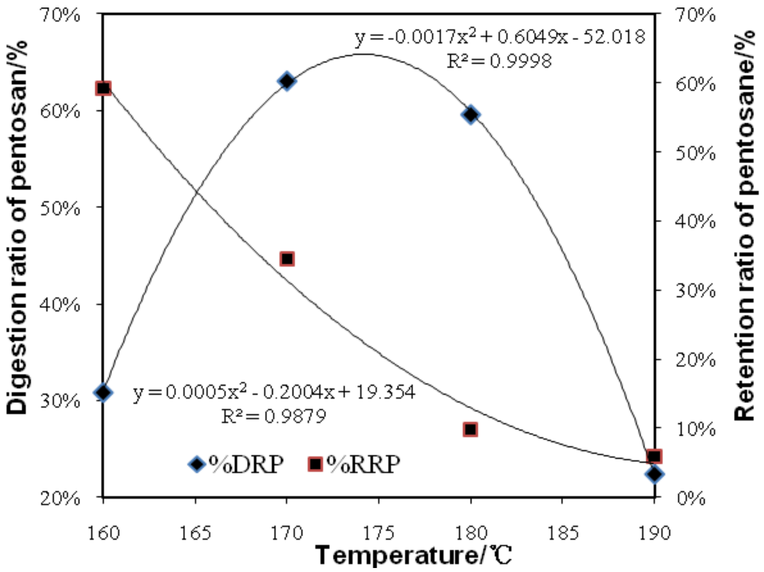

Fig. 7. Effect of reaction temperature on degradation of hemicellulose

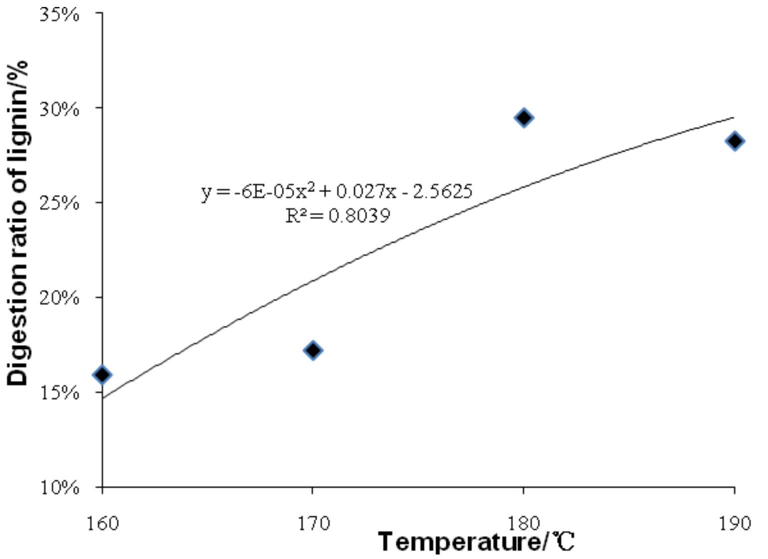

Fig. 8. Effect of reaction temperature on degradation of lignin

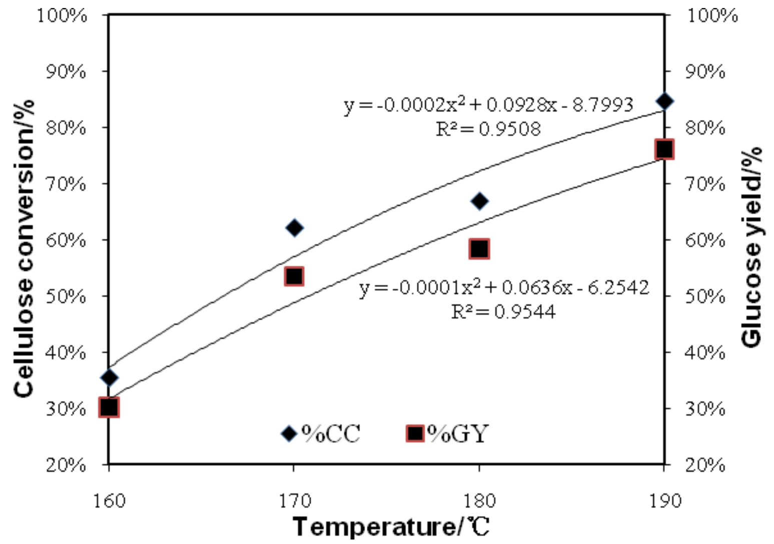

Fig. 9. Effect of reaction temperature on cellulose conversion and glucose yield

Throughout the temperature range considered, the data showed good agreement with the fitting curve. The retention ratio of pentosan declined with the increase of temperature. These data indicated that the degradation of hemicellulose held a dominant position in the temperature range of 160 ^{o}C to 180 ^{o}C, while following with conversion of pentosan. The degraded hemicellulose was converted into fermentation inhibitors in the sulfite pretreatment (A total of 70% to 95% of pentosan (%DRP plus %RRP) could be detected in the spent liquor and substrate). The detected pentosan from the spent liquor and substrate were 6% and 22% at the reaction temperature of 190 ^{o}C, which meant that over 70% of the pentosan had been converted into other chemicals (such as furfural, formic acid and levulinic acid) in the higher temperature pretreatment. Such findings support a pessimistic outlook concerning the enzymatic hydrolysis.

The degradation of lignin was aggravated at higher temperature, as Fig. 8 demonstrated. It is supposed that the degradation of hemicellulose may promote lignin dissolution from the lignin carbohydrate complexes. Lignin condensation at higher temperature was the main cause of the minor change of digestion of lignin at temperatures above 180 °C. It is found that the %CC and %GY climbed throughout the full temperature range.

The degradation of lignin and hemicellulose eliminated the hindrance to the enzymatic hydrolysis of cellulose and prevented the ineffective adsorption of enzyme to the hemicellulose, so that more active sites were created for cellulase to break down cellulose into oligosaccharide and cellobiose. The conversion of cellulose was higher than the other process, but more inhibitors generated from the glucose and pentosan during the higher temperature process would bring more trouble to the following fermentation test.

Effects of Retention Time on Pretreatment of Wheat Straw

The effects of the retention time on the component separation and saccharification were considered. In Fig. 10, the fitting curves of the %DRP and %RRP of pentosan vs. retention time show a downward trend with longer retention time. In these trials the reactions of hemicellulose degradation, the dissolution of pentosan, and hemicellulose conversion were activated at the same time.

The pentosan yield (%DRP plus %RRP) declining from 83% at 10 min to 63% at 40 min indicated that the extension of retention time favored hemicellulose conversion and enzymatic hydrolysis. After 40 min of retention, about 37% of pentosan was converted into inhibitors and other chemicals not expected to be favorable for the following fermentation test.

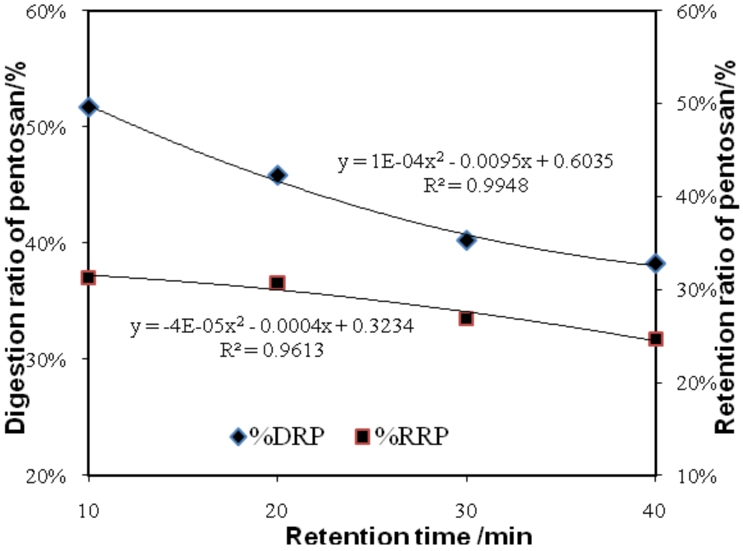

Fig. 10. Effect of retention time on degradation of hemicellulose

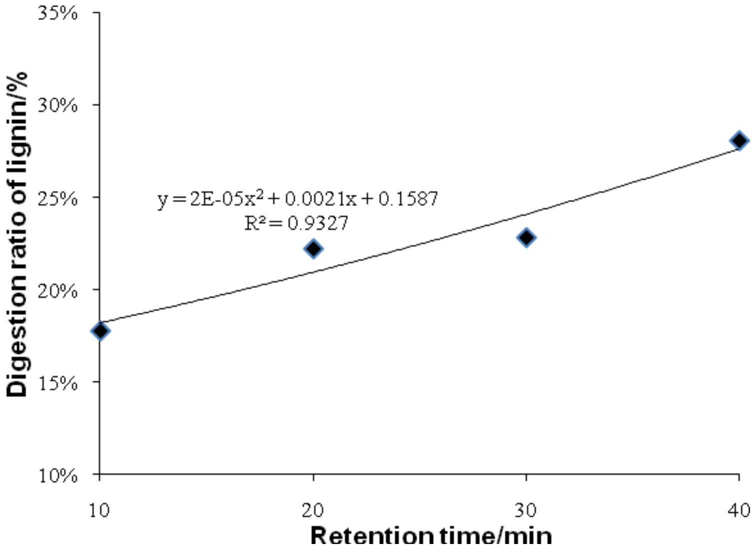

Fig. 11. Effect of retention time on degradation of lignin

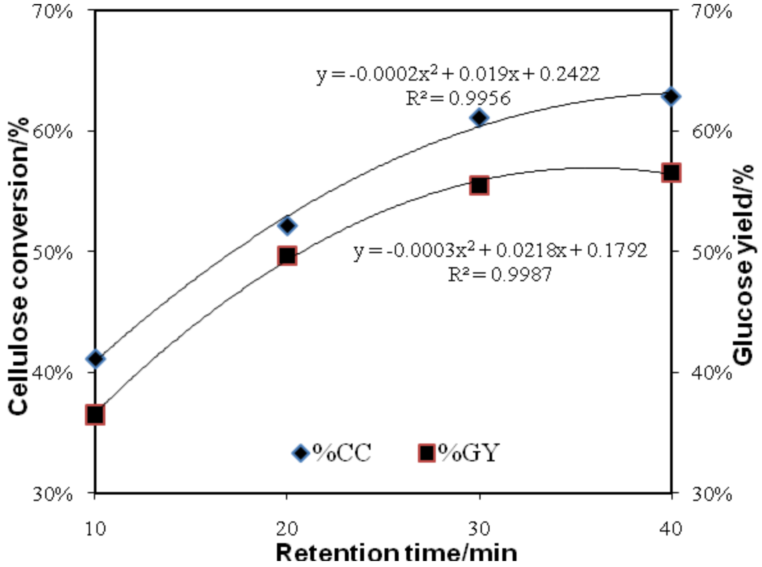

Fig. 12. Effect of retention time on cellulose conversion and glucose yield

In Fig.11, the lignin digestion ratio was promoted from 18% to 28%, which indicated that a relatively longer retention time would promote the dissolution of lignin. The cellulose conversion and glucose yield were plotted against the retention time in Fig.12. Comparing the two levels of 10 min and 40 min respectively in Fig.12, one can conclude that retention time had a positive effect on the cellulose conversion and glucose yield. This is all related to the degradation of hemicellulose and lignin during the pretreatment. From the fitting curves for %CC and %GY, the increase of cellulose conversion and glucose yield was easily observed after 30 min. The condensation of lignin under the high pressure, high temperature, and acidic conditions may block the substrate from the further interactions with cellulase.

Verification under the Optimal Condition

Taking saccharification efficiency, higher pentosan yield, and weakly acidic process into account, with the nonlinear regression analysis from all the tests, the optimal condition was selected: reaction temperature 180 °C, retention time 20 min, sodium bisulfite charge 3%, and sulfuric acid charge 1.48%. The following results can be found from Fig.13: 45.5% of pentosan, 24.5% lignin, and 3.3% glucan in the wheat straw were in the liquor in the verification test, while 25.0% pentosan, 75.5% lignin, and 79.6% glucan still existed in the substrate. Enzymatic hydrolysis was run with enzyme loading of about 5 FPU cellulase plus 4 CBU β-glucosidase per gram of o.d. substrate. After hydrolysis for 48 h, 29.57 g of glucose, corresponding to 90.9% of the glucose in the substrate and 72.4% glucan of the material, was determined. The sum of pentosan in liquor and substrate was 70.5%, which signified that about 30% of pentosan was converted into furfural or hydroxymethyl furfural during the acidic high temperature process. The remaining 75.5% lignin in the substrate could be collected for further utilization through solid-liquid separation after enzymatic hydrolysis.

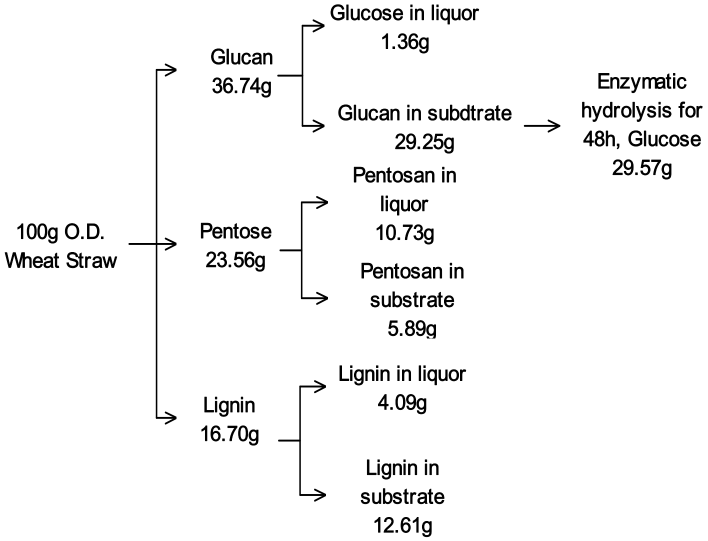

Fig.13. Analysis of flow direction of wheat straw components

CONCLUSIONS

1. The results of $L_{16}(4^4)$ orthogonal designed experiments demonstrated that: within the research scope, the saccharification efficiency can be improved through elevating the temperature, increasing the charge of sodium bisulfite or sulfuric acid, or extending the retention time. Degradation and dissolution of lignin and hemicellulose during the process play the key roles in the improvement of component separation and saccharification. The digestion ratios of lignin and pentosan can be raised by the methods above, but the further conversion of pentosan is running at the same time. The optimal condition for pretreatment is with a bisulfite charge of 3%, sulphuric acid charge 1.48% on untreated o.d. wheat straw, with temperature 180 °C for 20 min. After 48 h enzymatic hydrolysis, a glucose yield of 72.4% was achieved, based on the theoretical yield of untreated wheat straw.

2. Sulfite pretreatment can effectively separate wheat straw into three main components: cellulose, pentosan and lignin, overcoming the recalcitrance of lignocellulosic material in the enzymatic hydrolysis and reaching a satisfactory level of cellulose conversion.

ACKNOWLEDGMENTS

The authors are grateful for the financial support of Tianjin colleges & universities Science & Technology Fund, Grant No. 20080522, and State Key Lab of Pulp and Paper open fund, South China University of Technology, Grant No. 200924.

REFERENCES CITED

Burns, D. S., Ooshima, H., and Converse, A. O. (1989). "Surface area of pretreated lignocellulosics as a function of the extent of enzymic hydrolysis," *Applied Biochemistry and Biotechnology*, 20-21(1), 79-94.

Caldeira, K., Jain, A. K., and Hoffert, M. I. (2003). "Climate sensitivity uncertainty and the need for energy without CO_2 emission," *Science* 299(5615), 2052-2054.

Caufield, D. E., and Moore, W. E. (1974). "Effect of varying crystallinity of cellulose on enzymic hydrolysis," *Wood Sci.* 6(4), 375-379.

Chang, V. S., and Holtzapple. M. (2000). "Fundamental factors affecting biomass enzymatic reactivity," *Applied Biochemistry and Biotechnology* 84(86), 5-37.

Cowling, E. B., and Kirk, T. K. (1976). "Properties of cellulose and lignocellulosic materials as substrates for enzymatic conversion processes," *Biotechnology and Bioengineering Sympoium* 6, 95-123.

Demain, A. L., Newcomb, M., and Wu, J. H. D. (2005). "Cellulase, clostridia, and ethanol," *Microbiology and Molecular Biology Reviews,* 69(1), 124-154.

Emmel, A., Mathias, A. L., Wypych, F., and Ramos, L. P. (2003). "Fractionation of *Eucalyptus grandis* chips by dilute acid-catalyzed steam explosion," *Bioresource Technology* 86(2), 105-115.

Fan, L. T., Lee, Y. H., and Beardmore, D. H. (1980). "Mechanisms of the enzymatic hydrolysis of cellulose: Effects of major structural features of cellulose on enzymatic hydrolysis," *Biotechnology and Bioengineering.* 22(1), 177-199.

Farrell, A. E., Plevin, R. J., Turner, B. T., Jones, A. D., O'Hare, M., and Kammen, D. M. (2006). "Ethanol can contribute to energy and environmental goals," *Science* 311(5760), 506-508.

Grohmann, K., Mitchell, D. J., Himmel, M. E., Dale, B. E., and Schroeder, H. A. (1989). "The role of ester groups in resistance of plant cell wall polysaccharides to enzymatic hydrolysis," *Applied Biochemistry and Biotechnology* 20(21), 45-61.

Hinman, N. D., Schell, D. J., Riley, D., Bergeron, P. W., and Walter, P. J. (1992). "Preliminary estimate of the cost of ethanol-production for SSF technology," *Applied Biochemistry and Biotechnology* 34(1), 639-649.

Kong, F., Engler, C. R., and Soltes, E.J. (1992). "Effects of cell-wall acetate, xylan backbone, and lignin on enzymic hydrolysis of aspen wood," *Applied Biochemistry* *and Biotechnology* 34(35), 23-35.

Lee, D., Yu, A. H. C., and Saddler, J. N. (1995). "Evaluation of cellulase recycling strategies for the hydrolysis of lignocellulosic substrates," *Biotechnology and Bioengineering* 45(4), 328-336.

Lethers, T. D. (2003). "Bioconversion of maize residues to value-added coproducts using yeast-like fungi," *FEMS Yeast Research* 3(2), 133-140.

Philippidis, G. P., and Smith, T. K. (1995). "Limiting factors in the simultaneous saccharification and fermentation process for conversion of cellulosic biomass to fuel ethanol," *Applied Biochemistry and Biotechnology,* 51-52(1), 117-124.

Polcin, J., and Bezuch, B. (1977). "Investigation on enzymatic hydrolysis of lignified cellulosic material," *Wood Science Technology*. 11(4), 275-290.

Puri, V. P. (1984). "Effect of crystallinity and degree of polymerization of cellulose on enzymatic saccharification," *Biotechnology Bioengineering* 26(10), 1219-1222.

Ragauskas, A. J. (2006). "The path forward for biofuels and biomaterials," *Science* 311(5760), 484-489.

Saddler, J. N. (1993). "Introduction," *Bioconversion of Forest and Agricultural Plant Residues*, *Biotechnology in Agriculture*, No. 9, CAB International, UK, 1-11.

Sasaki, T., Tanaka, T., Nanbu, N., Sato, Y., and Kainuma, K. (1979). "Correlation between X-ray diffraction measurements of cellulose crystalline structure and the susceptibility of microbial cellulase," *Biotechnol. Bioeng.* 21(6), 1031-1042.

Schwalb, W., Brownell, H. H., and Saddler, J. N. (1988). "Enzymatic hydrolysis of steam treated aspen wood: Influence of partial hemicellulose and lignin removal prior to pretreatment," *Wood Chemistry and. Technology* 8(4), 543-560.

Wang, G. S., Pan, X. J., Zhu, J. Y., and Gleisner, R. (2009). "Sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL) for robust enzymatic saccharification of hardwoods," *Biotechnology Progress* 25(4), 1086-1093.

Wingren, A., Galbe, M., and Zacchi, G. (2003). "Techno-economic evaluation of producing ethanol from softwood: comparison of SSF and SHF and identification of bottlenecks," *Biotechnology Progress* 19(4), 1086-1093.

Yu, J. R., Zhang, Z., and Chi, C. C. (2007). "A rapid determination method for the pentosan in pre-extraction liquor from eucalyptus chips," *China Pulp & Paper*, 26(11), 10-13.

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

Zhu, J. Y., Pan, X. J., Wang, G. S., and Gleisner, R. (2009). "Sulfite pretreatment for robust enzymatic saccharification of spruce and red pine," *Bioresource Technology* 100, 2411-2418.

Zhu, J. Y., Zhu, W., O'Bryan, P., Dien, B. S., Tian, S., Gleisner, R., and Pan, X. J. (2010). "Ethanol production from SPORL-pretreated lodgepole pine: Preliminary evaluation of mass balance and process energy efficiency," *Applied Microbiology and Biotechnology* 86, 1355-1365.

Article submitted: October 24, 2010; Peer review completed: December 6, 2010; Revised version accepted: March 8, 2011; Published: March 10, 2011.