Efecto del pretratamiento con hidróxido de sodio a baja temperatura sobre la composición química y la hidrólisis enzimática del abeto

Effect of sodium hydroxide pretreatment at low temperature on chemical composition and enzymatic hydrolysis of spruce

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Key words: spruce, pretreatment, sodium hydroxide, enzymatic hydrolysis.

RESUMEN. La disponibilidad de azúcares fermentables es un factor que limita la producción a gran escala de productos biológicos como el bioetanol. Por eso, los procesos para producir azúcares se están desarrollando a partir de materiales lignocelulósicos, mediante la hidrólisis enzimática. Sin embargo, la fracción celulósica de estos materiales no está fácilmente accesible a las enzimas hidrolíticas, por lo que se requiere una etapa de pretratamiento para lograr una hidrólisis eficiente. Muchos procesos se investigan para la realización de este pretratamiento. El empleo de hidróxido de sodio está entre los métodos promisorios. En el presente trabajo, se investigó el efecto del pretratamiento con hidróxido de sodio sobre la composición química y la hidrólisis enzimática posterior del abeto. Se analizó una especie nativa de abeto, obtenida de los bosques que rodean la ciudad de Boras en Suecia, según los métodos analíticos estándares de biomasa (NREL) para conocer las fracciones de carbohidratos y lignina. La madera se trató químicamente mediante una disolución de hidróxido de sodio (7 % p/p) con una relación sólido-líquido de 5 % (p/v), a una temperatura de 0 °C por 0,5; 1, 2 y 3 h. En la hidrólisis enzimática se emplearon las enzimas comerciales celulasas (Celluclast 1,5 L, Novozyme, Denmark) y β-glucosidasa (Novozyme 188, Novozyme, Denmark) con actividades de 30 FPU y 50 UI por gramo de madera, respectivamente. Los pretratamientos provocaron cambios en la composición del material. Se obtuvo alrededor de un 98 % de recobrado de los carbohidratos iniciales, lo que indicó una hidrólisis muy baja. Los xilanos fueron los componentes más afectados por los pretratamientos. La mayor remoción de xilanos fue casi del 50 % y ocurrió al utilizar la disolución de hidróxido de sodio por 3 h. Se analizó también, el perfil de azúcares liberados. El rendimiento de la hidrólisis enzimática aumentó como resultado de los pretratamientos aplicados. Se pudo alcanzar un rendimiento de glucosa cercano a 40 %.

ABSTRACT. The availability of fermentable sugars is a limiting factor for large-scale production of biological products such as bioethanol. Therefore, processes to produce sugars are being developed from lignocellulosic materials by enzymatic hydrolysis. However, the cellulose fraction are not readily accessible for the hydrolyzing enzymes and an efficient hydrolysis requires pretreatment. Several processes have been investigated for this pretreatment. Pretreatment of lignocelluloses with NaOH is among the promissing methods. In the present work, the effect of NaOH pretreatment at low temperature on chemical composition and subsequent enzymatic hydrolysis of

spruce was investigated. A native spruce specie obtained from the forest around Borås city in Sweden was used in an the experiments. This wood was analyzed for carbohydrate and lignin fractions according to NREL methods. The wood was chemically pretreated using 7 % (w/w) sodium hydroxide solution with 5 % (w/v) solid content at 0 °C for 0.5, 1, 2 and 3 h. Commercial enzymes, cellulase (Celluclast 1.5 L, Novozyme, Denmark) and β-glucosidase (Novozyme 188, Novozyme, Denmark) were used in the enzymatic hydrolysis with activities of 30 FPU and 50 IU per gram of wood, respectively. The pretreatments changed the material composition. It was a very low loss of carbohydrate, about 98 % recovery, suggesting no significant carbohydrate hydrolysis. Xylans were the most affected by the pretreatments. The largest xylan removal was almost 50 %, using sodium hydroxide solution for 3 h. The profile of released sugars were also analyzed and compared. An improvement of enzymatic hydrolysis yield was observed as a result of the applied pretreatments, near 40 % glucose yield could be achieved.

INTRODUCTION

Energy consumption has increased steadily as world population has grown and more countries have become industrialized. Processes that enable utilization of renewable resources are becoming increasingly important as well as the amount of fossil resources reducing continuously and the environmental risk, caused by wastes produced from the use of fossil resources, is increasing.

Biomass is commonly recognized as one of the most important renewable resources. Ethanol, a clean and renewable energy source, which can be produced through fermentation, has drawn much attention from governments and researchers.[1] However, fermentative production of ethanol has been current limited using starch-based maize and sugar cane juice technologies because of shortage and high cost of raw materials. A potential method for low-cost ethanol fermentative production is the use of lignocellulosic materials. Over the last decades, lignocellulosic materials have been used as substrates for bioconversion into sugars, which then can be fermented to food additives, single cell protein, fuels and chemicals.[2]

The low susceptibility of the native lignocellulosic materials to hydrolysis can be overcome by physical, chemical or biological pretreatments to make the polysaccharide fractions accessible to enzymes, since polysaccharides are associated with lignin that difficults its hydrolysis.

Alkali pretreatment with NaOH has several beneficial effects: (I) efficient delignification, (II) open structures, (III) removal of hemicelluloses and (IV) ruptures of lignin-carbohydrate bonds. Saccharification is enhanced as a result of delignification and structural swelling, increasing superficial area and reduction of the polymerization degree and crystallinity.[3-5] Those changes increase the biodegradability of cell walls due to cleavage of bonds between lignin and hemicellulose or lignin and cellulose.[6] The efficiency of NaOH pretreatment depends greatly on process conditions, e.g. temperature, concentration of NaOH, and time of treatment, as well as the inherent characteristics of the lignocellulose used.[7-9]

NaOH pretreatment processes can be classified as “high concentration” and “low concentration” processes. In low-concentration processes, typically 0.5 - 4 % of NaOH, high temperature and pressure are used, and no recycling of NaOH occurs. NaOH at high temperatures disintegrates the lignin and hemicellulose and remove them from the solid phase. In high-concentration NaOH pretreatment, usually 6 to 20 % of NaOH, atmospheric pressure and low temperatures are used. The mechanism of this process occurs by dissolving cellulose, at least with 6 - 8 % NaOH solution at low temperatures and environmental pressure. In this process, lignin is not significantly removed from the cellulose.[10] NaOH pretreatments have been thoroughly studied in lignocellulosic materials at different temperatures. It has been proven that NaOH pretreatments at room temperature and cold temperatures need a long exposure time, just to produce enough dissolution of cellulose and hemicellulose. However, short times have been used at high temperature treatment since a lot of carbohydrates and lignin are dissolved in alkaline

solution for extended treatment.[11] Sodium hydroxide at cold temperatures has been studied to increase glucose conversion yield of spruce. The results indicated that enzymatic hydrolysis could be significantly improved by increasing the amount of sodium hydroxide up to 7 %.[11] However, the influence of pretreatment time at mild temperature on the enzymatic hydrolysis of spruce has not been studied, in order to reduce process time and energy consumption.

The objective of this research was to develop a novel pretreatment method for bioconversion of softwood. In this work, the effect of sodium hydroxide solution (7 %) at low temperature and different pretreatment times, just to improve enzymatic hydrolysis of spruce were investigated. The dissolution of spruce wood components, such as cellulose, hemicelluloses and lignin using the proposed approach was examined. Enzymatic hydrolysis of pretreated wood particles was compared with untreated one.

MATERIALS AND METHODS

Materials

Native spruce wood was obtained from the forest around Borås city in Sweden. The wood was debarked and ball milled (MM 400, Retsch GmbH, Hann, Germany) and then sieved to collect the fraction of 75 – 500 μm in size, which was used in all experiments. The chips were stored in a plastic bag prior to use. Commercial enzymes, cellulase (Celluclast 1.5 L, Novozyme, Denmark) and β-glucosidase (Novozyme 188, Novozyme, Denmark) were used in enzymatic hydrolysis.

Pretreatment

Oven dry wood chips were chemically pretreated using 7 % (w/w) sodium hydroxide solution. The solid wood-alkali solution ratio was 5/95 (w/v). Fiber bundles were treated at 0 °C for 0.5, 1, 2 and 3 h under atmospheric pressure. The wood suspensions in NaOH dissolution were equilibrated at the desired temperature before starting the pretreatment. The suspensions were agitated manually every 5 min in order to achieve an appropriate mixture. After pretreatments, the residual solids were washed with distilled water in order to remove undesired chemicals and neutralized to pH 7. Neutral samples were stored at 5 °C for further use.

Enzymatic hydrolysis

Enzymatic hydrolysis of pretreated and untreated wood fiber bundles was carried out at 5 % (w/v) of wood component based on dry weight in buffer citrate, using a shaker for 96 h at 200 r/min. The pH and temperature were adjusted to 4.8 and 45 °C, respectively. The mixtures were autoclaved at 121 °C for 20 min before adding the enzymes. Celluclast mixture and Novozym 188 with activities of approximately 30 FPU and 50 IU per gram of substrate were added for enzymatic hydrolysis. An excess of Novozym 188 was used to prevent cellobiose accumulation.[12] Hydrolyzates were sampled periodically for sugar analysis. Each data point was averaged from two replicas.

Analytical methods

Untreated and pretreated wood samples were analyzed for carbohydrate and lignin fractions according to NREL methods.[13-15] The acid-soluble lignin was measured by UV-vis spectroscopy at 205 nm. The dry weight content was measured using a convection drying oven at 105 °C, until constant weight. High performance liquid chromatography (HPLC) was used to quantify sugars. The HPLC (Alliance 2695, Waters, Milford, MA) was equipped with an RI detector (Waters 2414). An ion-exchange column (Aminex HPX-87H, Bio-Rad, Hercules, CA) at 60 °C, using 0.005

mol/L H_2SO_4 as eluent at flow rate 0.036 L/h, was utilized for the analysis of sugars. Composition values were calculated on dry weight basis. All experiments were carried out in two replicas, and all results were mean values. All the experimental results were processed using the software STATGRAPHICS Plus 5.1, just to perform analysis of variance, which allowed to compare results and determine if there were statistically significant differences among means. The Multiple Range Test was used to determine which means significantly different one another. The method used to discriminate among means was the Fisher's least significant difference (LSD) procedure. This method applies a multiple comparison procedure, just to determine statistically significant differences between means.

RESULTS AND DISCUSSION

Spruce composition

The chemical composition of wood fiber bundles before treatment was analysed (Table 1). Untreated spruce mainly consists of lignin, glucan, mannan and xylan. Only small amounts of arabinan and galactan were detected. These results agreed with those reported by other authors about carbohydrate and lignin content for softwood materials and also for spruce.[1,11,16] Obviously, this is a suitable material for ethanol production, since it contains a high amount of cellulose and mannan (57 %), whose hydrolyzates are easily fermentable to ethanol.

Pretreatment effects on wood composition

The chemical composition of wood fiber bundles after pretreatments were analyzed (Table 1). Applied pretreatments caused small changes on material composition. The four pretreatments produced very low glucan losses with a recovery of about 98 %, suggesting no significant carbohydrate hydrolysis. The low degrees of degradation of cellulose and hemicellulose do not favor enzymatic hydrolysis.[17] Xylans were the most affected components by pretreatments. The biggest xylan removal was almost 50 %, by using sodium hydroxide solution for 3 h.

Table 1. Chemical composition of pretreated and untreated spruce wood.

Time Treatment	Glucan (%)	Xylan (%)	Mannan (%)	ASL[1] (%)	AIL[2] (%)	Solid yield (%)
0.5 h	47.99 ± 1.26	3.04 ± 0.47	12.33 ± 0.94	0.50 ± 0.02	27.07 ± 0.35	90.59 ± 0.98
1 h	48.05 ± 1.21	2.88 ± 0.59	12.29 ± 1.61	0.48 ± 0.10	27.04 ± 0.32	90.42 ± 1.22
2 h	48.18 ± 0.89	2.85 ± 0.55	12.21 ± 0.10	0.47 ± 0.02	27.03 ± 0.17	90.12 ± 0.71
3 h	48.27 ± 1.63	2.79 ± 0.27	12.18 ± 1.63	0.47 ± 0.10	26.98 ± 0.27	89.81 ± 0.89
Untreated	44.37 ± 1.24	4.90 ± 0.45	12.60 ± 1.69	0.56 ± 0.07	28.20 ± 0.46	-

[1] Acid-soluble lignin, [2] Acid-insoluble lignin.

The effect of pretreatment time on the chemical composition of wood was also analysed (Table 1). The higher pretreatment time the higher polysaccharide and lignin removal and particularly, hemicellulose loss. Although there was cellulose loss, was found negligible and lesser than hemicellulose and lignin ones. Three hours of pretreatment

allowed reaching a maximum delignification of about 14 %. It is known that NaOH generally breaks lignin-lignin bonds and dissolves lignin.[18] This phenomenon occurs even under mild conditions and short times of pretreatment. Since hemicellulose forms acetal bonds with lignin, solubilization of hemicellulose is expected even under mild conditions.[19] The largest mannan removal was around 13 %. This happened by using sodium hydroxide solution for 3 h. These results were not unexpected since it is known that xylan of hemicellulose can be extracted quite well in alkaline environment, while glucomannan can hardly be extracted in acid environment and needs a stronger alkaline environment than xylan, just to be extracted.[20-22] Xylan appears to be the part that can be extracted easier. The F-test in the analysis of variance that there were significant differences amongst the means of sugars and lignin recovery in the solid fraction at 95.0 % confidence level. The Fisher's least significant difference (LSD) procedure indicated that there were statistically significant differences between glucan, xylan and lignin amounts of untreated and pretreated materials. This test also revealed no statistically significant differences between composition of pretreated materials and among mannan recovery of all considered materials at 95.0 % confidence level.

The effects of pretreatments on enzymatic hydrolysis of cellulose

Spruce fiber treated with NaOH at 0 °C for 0.5, 1, 2 and 3 h were subjected to enzymatic hydrolysis. The results revealed that pretreatment affected enzymatic hydrolysis (Fig. 1). Enzymatic hydrolysis efficiency increased up to 20 % in comparison with untreated wood. Up to 40 % theoretical glucose yield could be achieved from enzymatic hydrolysis of treated fiber for four days with the cited enzymes loading amounts. Results indicated that a small improvement on enzymatic hydrolysis efficiency was found in general when the pretreatment time was increased from 0.5 to 3 h while other experimental parameters remain the same (Fig. 1). Around 7 % of efficiency improvement was obtained with time increment, this might happens because pretreatment times were very close between one each other. Although all pretreatments improved enzymatic convertibility of cellulose, effectiveness was low, may be NaOH pretreatment at cold temperature needs a longer exposure time than those ones from this investigation. These analysis support the hypothesis that achieving satisfactory substrate cellulose conversion requires partial degradation of cellulose and removal of hemicellulose and lignin.[17]

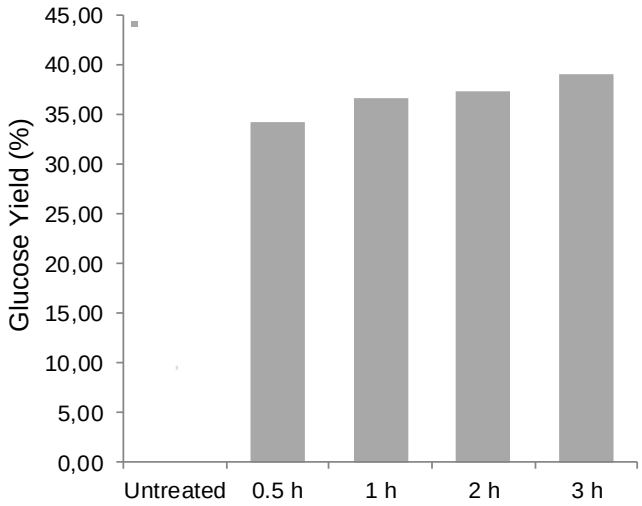

Fig. 1. *Pretreatment time effects on bioconversion of cellulose.*

The F-test in the analysis of variance showed significant differences among the means of glucose yield, reached for 96 h of enzymatic hydrolysis at 95.0 % confidence level. The Fisher's least significant difference (LSD) procedure indicated statistically significant differences between glucose yield of the untreated and pretreated materials. In contrast, this test revealed no statistically significant differences between glucose yield of the pretreated materials for 1 h and 2 h at 95.0 % confidence level.
The enzymatic hydrolysis trend was the same for the different pretreatments (Fig. 2). Two distinct bioconversion stages of cellulose to glucose by enzymatic hydrolysis were visualized. An initial quick enzymatic hydrolysis step occurred in the first 24 h, then followed by a lesser fast enzymatic hydrolysis reaction. Even though, the enzymatic hydrolysis rate remained increasing for all the hydrolysis time. Around 60 % of the total glucose was produced in the first day and the other 40 % in the three remaining ones. The rate of hydrolysis is usually very high firstly, and then decreases in the later stages. The specific surface area, or accessible surface area per gram of substrate, sharply increases during the initial stage.[23] However, it was shown that the cellulose surface area is not a major limiting factor for hydrolysis of pure cellulose [24]. In other words, the slowdown of hydrolysis in the later stages is not due to a lack of associable surface area, but difficulty in hydrolysis of the crystalline part of cellulose. Therefore, one may expect a lower rate of the hydrolysis after hydrolysis of the amorphous cellulose.[24]

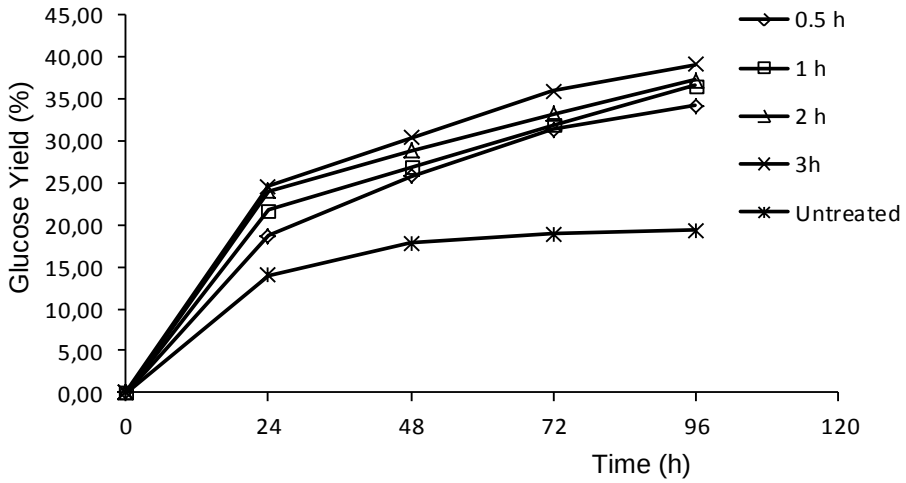

Fig. 2. *Effect of the time on cellulose bioconversion by enzymatic hydrolysis reaction of untreated and pretreated spruce wood.*

The largest value of convertibility was reached with the pretreated sample by using sodium hydroxide dissolution for 3 h. This pretreatment produced the biggest hemicellulose removal. The near complete separation of hemicellulose from cellulose is favorable, not only to the enzymatic hydrolysis of cellulose but also to the biological conversion of hemicellulose, due to lack of efficient technologies for cofermenting xylose and glucose.[17] Some previous studies reported the effect of sodium hydroxide as a pretreatment at cold temperatures on enzymatic hydrolysis. The results indicated that cellulose dissolution could be significantly improved by chemical treatment using sodium hydroxide at cold temperature, achieving satisfactory substrate cellulose bioconversion.[11,25-28] This treatment can slightly remove lignin, hemicelluloses and cellulose in lignocellulosic materials, disrupting connections between hemicelluloses, cellulose and lignin, and changing the structure of treated biomass, just to make cellulose more accessible to hydrolytic enzymes.[11]

Maximum glucose yield reached for pretreated spruce was more than twice the reported ones by Zhao *et al*. for pretreated spruce at 23 and 60 °C, when other conditions remained almost the same. Though, glucose conversion achieved in the cited study for pretreated spruce at colder temperature (– 15 °C) was higher than the ones from this work at 0 °C. This result reveals that treatment temperature greatly affect enzymatic hydrolysis, and cold temperature significantly increases the enzymatic hydrolysis efficiency of spruce. However, it has to be pointed out that the pretreatment time used in this investigation (3 h) was eith times smaller than the used by Zhao *et al*. (24 h), with the subsequent energy saving during cooling process.

CONCLUSIONS

The composition of spruce was determined before and after treatments in order to evaluate the structural change caused on wood. The applied pretreatments caused small changes on the composition of the material, guaranteeing high glucan preservation of about 98 %. Maximum delignification was of around 14 %. Xylans were the most affected components, removing almost 50 % of the initial amount.

In contrast, all pretreatment improved enzymatic convertibility of cellulose compared with that of the untreated wood, sodium hydroxide treatment for 3 h was the best condition of this study. Almost 40 % theoretical glucose yield could be achieved by using this pretreatment condition. However, small improvement was found with the increase of the pretreatment time.

REFERENCES

1. Sun Y, Cheng J. Hydrolysis of lignocellulosic materials for ethanol production: A review. Bioresource Technol. 2002;83(1):1-11.
2. Krishna HS, Chowdary GV, Reddy SD, Ayyanna C. Simultaneous saccharification and fermentation of pretreated Antigonum leptopus (Linn) leaves to ethanol. J Chem Technol Biotechnol. 1999;74(11): 1055-60.
3. Gharpuray MM, Fan LT, Lee YH. Caustic pretreatment study for enzymatic hydrolysis of wheat straw. Wood and agricultural residues: research on use for feed, fuels, and chemicals: proceedings, Conference, Kansas City, Missouri, Sept 12-17, 1982 / spon. American Chemical Society; ed. E.J. Soltes. New York: Academic Press. Wood Agric Residue. 1983: 369-89.
4. Fan LT, Gharpuray MM, Lee YH. Cellulose Hydrolysis. 1st ed. Berlin, Germany: Springer-Verlag: 1987:p.1-68.
5. Fox DJ, Gray PP, Dunn NW, Mardsen WL. Factors affecting the enzymatic susceptibility of alkali and acid pretreated sugar-cane bagasse. J Chem Techn Biotechnol. 1987;40:117-32.
6. Spencer RR, Akin DE. Rumen microbial degradation of potassium hydroxide-treated coastal bermuda-grass leaf blades examined by electron microscopy. J Anim Sci. 1980; 51(5):1189-96.
7. Zhao Y, Wang Y, Zhu J, Ragauskas A, Deng Y. Enhanced enzymatic hydrolysis of spruce by alkaline pretreatment at low temperature. Biotechnol Bioeng. 2008; 88:797-824.
8. Sharma K, Kuhar S, Kuhad R, Bhat P. Combinatorial approaches to improve plant cell wall digestion: Possible solution for cattle feed problems. Lignocellulose Biotechnology: Future Prospects, Kuhad, R.C., and Singh, A., editores. IK International Publishing House Pvt. Ltd., New Delhi: 2007:p.120-80.
9. Wanapat M, Sundstøl F, Garmo TH. A comparison of alkali treatment methods to improve the nutritive value of straw. Anim Feed Sci Technol. 1985;12:295-309.

10. Mirahmadi K, Mohseni M, Jeihanipour A, Karimi K, Taherzadeh MJ. Alkaline pretreatment of spruce and birch to improve bioethanol and biogas production. BioResources. 2010; 5(2): 928-38.
11. Zhao Y, Wang Y, Zhu JY, Ragauskas A, Deng Y. Enhanced Enzymatic Hydrolysis of Spruce by Alkaline Pretreatment at Low Temperature. Biotechnol Bioeng. 2007;99:1320-28.
12. Emmel A, Mathias AL, Wypych F, Ramos LP. Fractionation of Eucalyptus Grandis chips by dilute acid-catalysed steam explosion. Bioresource Technol. 2002;86:105-15.
13. Ehrman T. Standard method for determination of total solids in biomass, Laboratory Analytical Procedure No. 001, National Renewable Research Laboratory (NREL). Golden. CO. 1994. [Consulted december 6th, 2010]. Available at: www.nrel.gov
14. Ruiz R. and Ehrman T. Determination of carbohydrates in biomass by high performance liquid chromatography, Laboratory Analytical Procedure No. 002, National Renewable Research Laboratory (NREL). Golden. CO. 1996. [Consulted december 6th, 2010]. Available at: www.nrel.gov
15. Templeton D. and Ehrman T. Determination of acid-insoluble lignin in biomass, Laboratory Analytical Procedure No. 003, National Renewable Research Laboratory (NREL). Golden. CO. 1995. [Consulted december 6th, 2010]. Available at: www.nrel.gov
16. Galbe M, Zacchi G. A review of the production of ethanol from softwood. Appl Microbiol Biotechnol. 2002;59(6):618-28.
17. Zhu JY, Wang GS, Pan XJ, Gleisner R. Sulfite pretreatment (SPORL) for robust enzymatic saccharification of spruce and red pine. Bioresource Technology. 2008;100:2411-18.
18. Scalbert A, Monties B. Comparison of wheat straw lignin preparations. II. Straw lignin solubilization in alkali. Holzforschung. 1986;40(4): 249-54.
19. Macris BJ, Christakopulos PF, Kekos D, Koullas DP and Koukios EG. Direct bioconversion of pretreated straw to ethanol. Energy from Biomass CEC Contractors' Meeting Proc. (G. Grassi, D. Pirrwitz and H. Zibetta, eds). 1988: p. 356-66.
20. Balaban M, Ucar G. The effect of the duration of alkali treatment on the solubility of polyoses. Turk J Agric Forest. 1999;23:667-71.
21. Fengel D, Wegener G. Wood: Chemistry, Ultrastructure, Reactions. Walter de Gruyter, Berlin, Germany. 1984: 132-181.
22. Lawther JM, Sun R, Banks WB. Effects of extraction conditions and alkali type on yield and composition of wheat straw hemicellulose. J Appl Polym Sci. 1996; 60:1827-37.
23. Taherzadeh MJ, Karimi K. Pretreatment of Lignocellulosic Wastes to Improve Ethanol and Biogas Production: A Review.Int J Mol Sci. 2008;9:1621-1651.
24. Fan LT, Lee Y, Beardmore DH. Mechanism of the enzymatic hydrolysis of cellulose: Effects of major structural features of cellulose on enzymatic hydrolysis. Biotechnol. Bioeng. 1980; 22: 177-199.
25. Zhou JP, Zhang L, Cai J, Shu H. Cellulose microporous membranes prepared from NaOH/Urea aqueous solution. J Membr Sci. 2002;210(1):77-90.
26. Zhou JP, Zhang L, Cai J. Behavior of cellulose in NaOH/urea aqueous solution characterized by light scattering and viscometry. J Polym Sci B Polym Phys. 2004;42(2):347-53.
27. Zhou JP, Qin Y, Liu SL, Zhang L. Homogenous synthesis of hydroxyethyl cellulose in NaOH/urea aqueous solution. Macromol Biosci. 2006;6(1):84-9.

28. Cai J, Zhang L. Rapid dissolution of cellulose in LiOH/urea and NaOH/urea aqueous solutions. Macromol Biosci. 2005;5(6):539-48.