EFFECT OF GLYCEROL PREATREATMENT ON COMPONENT RECOVERY AND ENZYMATIC HYDROLYSIS OF SUGARCANE BAGASSE

CARLOS MARTÍN,[*,**] JÜRGEN PULS,[*] BODO SAAKE[***] and
ANDREAS SCHREIBER[***]

[*]*vTI – Institute for Wood Technology and Wood Biology, Hamburg, Germany*
[**]*Department of Chemistry and Chemical Engineering,
University of Matanzas, Cuba*
[***]*Hamburg University, Wood Science Centre, Chemical Wood Technology, Germany*

Sugarcane bagasse was pretreated with glycerol (40-80% (w/w)) either in the presence of NaOH or H_2SO_4, or without any chemicals, at 190 °C, 10% biomass load, for 1 to 4 hours. The pretreatments resulted in cellulose-enriched fibres and different degrees of xylan and lignin solubilisation. Cellulose recovery was higher in pretreatments with NaOH addition and without any chemicals, than in those with H_2SO_4. Xylan solubilisation was almost complete in all H_2SO_4-assisted treatments whereas, in the other experiments, it decreased with the increase in glycerol concentration and increased with the pretreatment time. Lignin solubilisation increased proportionally with glycerol concentration. Under all conditions applied, the pretreatment improved the enzymatic hydrolysis of cellulose. The highest overall cellulose convertibilities were achieved in the glycerol pretreatments without other chemicals (85-94%). Convertibility decreased with time in acidic pretreatments, and increased slightly for the other ones. Enzymatic conversion of the xylan retained in the fibres was also detected.

Keywords: sugarcane bagasse, glycerol, pretreatment, enzymatic hydrolysis

INTRODUCTION

Glycerol is a by-product of biodiesel production. Although crude glycerol can be further upgraded to pharmaceutical, cosmetic and food industry, the extensive biodiesel production has already started to flood the market and drive the prices down.[1,2] The current 0.9-1.0 Mt world glycerol demand can be covered, and doubled, by the about 1.9 Mt generated by the European biodiesel industry.[3-5] Consequently, the development of new products and processes is required for dealing with the glycerol surplus. Different fermentative processes for glycerol conversion into 1,3-propanediol,[6-8] 3-hydroxypropionic acid,[7] citric acid,[6,9] lactic acid,[10] single-cell oil,[6] hydrogen and ethanol[11] are investigated. Intensive research is carried out, as well, on the production of the already known and completely new glycerol derivatives obtained by chemical synthesis.[5]

A potential use of glycerol that presents interest is in organosolv pulping, for fractionation of lignocellulosic materials and for improving the enzymatic hydrolysis of cellulose.[12] Organosolv pulping, based on the use of organic solvents, has been investigated as an alternative to alkaline kraft pulping[13] and, since it leads to a solid phase enriched in easily hydrolysable cellulose, it has potential as pretreatment for enzymatic hydrolysis. However, so far, the scope of organosolv pretreatment has been limited, due to the risk of high pressure operation and the use of highly volatile and flammable solvents. As a non-flammable compound, glycerol is attractive for organosolv processes, and the drawback of its energy-intensive recovery can be solved by transferring it into epoxy resins.[14]

Up to now, glycerol pulping has been investigated only for a few wood species and agricultural residues,[2,12,15] and there are no

reports on using it for sugarcane bagasse, which is a very abundant material in many tropical countries.[16,17] In previous reports, glycerol pulping has been assisted by alkaline catalysts,[12,18,19] while the effect of acid catalysts, known to improve the effectiveness of organosolv processes,[20] has not been tested in glycerol pretreatments. In this work, the effect of glycerol pretreatments – alkaline, acidic or aqueous – on the separation and recovery of sugarcane bagasse components, and on the enzymatic hydrolysis of cellulose is assessed.

MATERIALS AND METHODS
Materials
Sugarcane bagasse acquired from a sugar mill in Sudan was used. The material was kept under constant moisture and temperature conditions, in a climatization chamber. A portion of bagasse was milled to 1 mm particle size and used for raw material analysis. No previous milling was applied to the material used in pretreatment experiments.

Glycerol pretreatment
40, 60 or 80% (w/w) aqueous glycerol, containing either NaOH (0.3 g per 100 g mixture) or H_2SO_4 (0.94 g per 100 g mixture), or without any chemicals, was mixed with 60 g of bagasse, at a liquid-to-solid ratio of 10%, in 1 L stainless steel cylindrical containers mounted on a rotary autoclave, and treated at 190 °C for 1 to 4 hours

(Table 1). After that, the liquors were separated by vacuum filtration, and the pretreated solids were washed – first with aqueous glycerol, and then with abundant warm water. The pretreatment liquor and glycerol washes were stored in plastic containers at 4 °C, for analysis and further use, and the water washes were discarded. The pretreated solids were refined with a pulp refiner (Sprout Waldron, Spring, USA), air-dried at 20 °C and 60% humidity, and stored in plastic bags for chemical analysis and enzymatic hydrolysis tests.

Analytical methods
The chemical composition of both raw and pretreated bagasse was determined by analytical acid hydrolysis, followed by quantification of the sugars contained in hydrolysates, by borate complex ion exchange chromatography.[21] The sugars were separated on a MCI Gel CA08F (Mitsubishi) column at 60 °C, using a linear gradient of 0.3 to 0.9 M potassium borate buffer (pH 9.2), at a flow rate of 0.7 mL/min within 35 min. To allow spectrophotometric detection at 560 nm, derivatization with cuprum bicinchonate was performed.

The concentrations of glucose and xylose were used for calculating the content of cellulose and xylan, respectively. Acid-insoluble lignin was determined gravimetrically, whereas the soluble lignin was determined spectrophotometrically at 205 nm, on a UV/VIS spectrophotometer (LAMBDA 650, Perkin Elmer LAS, Rodgau, Germany).

Table 1
Experimental conditions

Experiment Nº	Glycerol concentration, % (w/w)	Treatment time, h	Chemical used
1	40	1	NaOH
2	40	1	H_2SO_4
3	40	1	-
4	60	1	NaOH
5	60	1	H_2SO_4
6	60	1	-
7	80	1	NaOH
8	80	1	H_2SO_4
9	80	1	-
10	60	2	NaOH
11	60	2	H_2SO_4
12	60	2	-
13	60	3	NaOH
14	60	3	H_2SO_4
15	60	3	-
16	60	4	NaOH
17	60	4	H_2SO_4
18	60	4	-

The extractive compounds were determined by accelerated Soxhlet extraction (ASE 200, Dionex, Sunnyvale, USA) using petroleum ether, an acetone/water mixture and water as solvents. The mineral components were determined as ash, after incineration of an aliquot of the material at 550 °C, in a muffle oven (Heraeus Instruments GmbH, Hanau, Germany). The sugar content in the pretreatment liquors was determined by posthydrolysis with 0.2 M H_2SO_4, followed by chromatographic analysis. All analyses were performed in triplicate.

Enzymatic convertibility

Approximately 1.08 g of the washed pretreated material was suspended in 25 mL of citrate buffer (pH 5.0). A commercial preparation of *Trichoderma reesei* cellulases (Celluclast 1.5 L) and a β-glycosidase preparation (Novozym 188) (Novozymes, Denmark) were added to a loading of 25 FPU/g DM and 0.46 CBU/mL, respectively. The reaction mixture was incubated in an incubation hood (Certomat HK, Sartorius AG, Göttingen, Germany) at 45 °C and 150 rpm, for 72 h. At the end of hydrolysis, glucose was quantified by HPLC, and the results were used for calculating the enzymatic convertibility of cellulose. All experiments were performed in triplicate.

RESULTS AND DISCUSSION
Chemical composition of bagasse

Chemical characterisation of the raw material confirmed the high potential of sugarcane bagasse for its conversion through the saccharification route. With a carbohydrate content exceeding 70% dry weight, including around 44% cellulose and 25% xylan (Table 2), bagasse is an excellent feedstock for fermentative processes. Furthermore, the chemical analyses of this Sudanese bagasse are in good agreement with the content of other sorts of bagasse produced in different countries, such as Cuba,[22,23] Brazil[17] and Mexico.[24]

Pretreatment at different glycerol concentrations

For investigating the influence of the glycerol load on the recovery of the main components and on the enzymatic hydrolysis of cellulose, a set of experiments was conducted at three different concentrations, for 1 hour (Experiments 1-9, Table 1). The effect of glycerol concentration on the solubilisation of bagasse components displayed different trends, depending on the chemical used for assisting the pretreatment process. As interpreted from the dry matter yield (Fig. 1A), solubilisation was enhanced by sulphuric acid and decreased by sodium hydroxide. The yield of solids, of 67-76% in the aqueous glycerol treatment, decreased to 53-63% in the presence of H_2SO_4, and increased to 81-90% in the presence of NaOH. On the other hand, unlike aqueous and alkaline glycerol treatments, acidic glycerol led to a decreased yield of solids with the increase of glycerol concentration.

Chemical analysis of the pretreated solids revealed that, as result of lignin and xylan solubilization, the cellulose content of all pretreated materials was higher than that of raw bagasse (Table 3). The content of cellulose and lignin in the cellulignin obtained in the H_2SO_4-assisted pretreatment was higher than in the pretreated solids produced by the other processes. Such a result is related to the high degree of xylan hydrolysis under acidic conditions, when that component was almost totally removed from the solids, independently of glycerol concentration in the reaction mixture. The cellulose content in the solids resulting from acidic pretreatment increased from around 65%, at the lowest glycerol concentration, to almost 72% at the highest glycerol concentration, while the lignin content decreased from 29 to 19%. In spite of the high cellulose content, the recovery chart reveals that cellulose was partially solubilised during the acidic pretreatment, especially at glycerol concentrations of 60 and 80% (Fig. 1B).

In pretreatments with NaOH or without chemicals, where xylan solubilisation was less intense than in the acidic process, cellulose was almost completely recovered in pulps (Fig. 1B), and its content was less affected by glycerol concentration (Table 3). Xylan solubilisation was higher in the aqueous pretreatment than in the alkaline one, and it decreased with increasing glycerol concentration in both processes (Fig. 2A). The decrease of solubilisation resulted in increases of xylan content in the fibres, from around 5 to around 12% in the neutral pretreatment, and from 17.6 to 25% – in the alkaline one (Table 3).

The increase of glycerol concentration in the reaction mixture led to a clear increase of delignification and to a decrease of xylan solubilisation (Figs. 2A and 2B), indicating that, at high concentrations, glycerol acts more selectively on lignin than on xylan. This is especially true for the alkaline pretreatment which, at 80% glycerol,

solubilised around half of the initial lignin and only about 8% xylan. Lignin solubilisation was noticeably affected by the type of chemical used for assisting the pretreatment. At 40% glycerol concentration, the highest lignin solubilisation (32.3%) was achieved in the presence of NaOH, whereas, when using 80% glycerol, the highest solubilisation (54.4%) was obtained in the H_2SO_4-assisted process.

Table 2
Chemical composition of bagasse (standard deviations shown in parentheses)

Component	Content, % (w/w)
Cellulose	43.8 (1.8)
Xylan	24.7 (1.2)
Arabinan	1.6 (0.0)
Galactan	0.5 (0.0)
Mannan	0.2 (0.0)
Lignin	22.6 (0.9)
Water extractives	1.6 (0.0)
Organic extractives	2.3 (0.0)
Ash	2.0 (0.0)

Table 3
Composition of the material treated under different glycerol concentrations
(standard deviations are shown in parentheses)

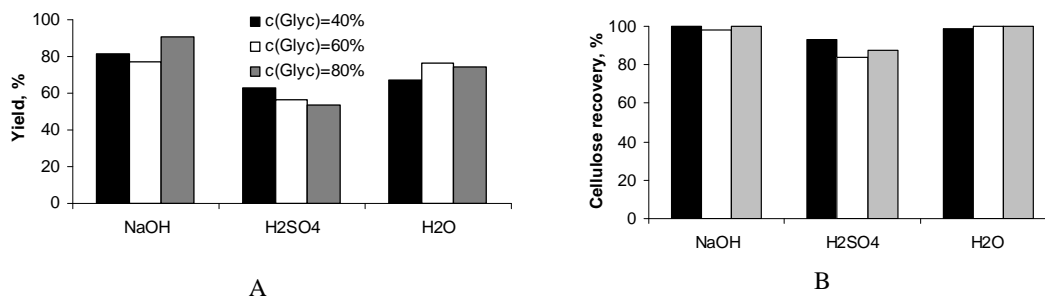| N^o	Cellulose, % (w/w)	Xylan, % (w/w)	Lignin, % (w/w)
1	58.0 (0.2)	17.6 (0.1)	18.8 (0.3)
2	65.1 (0.6)	1.0 (0.0)	29.4 (0.6)
3	64.2 (0.1)	4.9 (0.0)	25.8 (0.1)
4	55.6 (0.2)	20.5 (0.1)	17.8 (0.3)
5	65.4 (0.4)	0.6 (0.0)	30.0 (0.2)
6	65.0 (0.4)	6.6 (0.0)	22.2 (0.6)
7	53.3 (0.8)	25.0 (0.5)	12.9 (0.4)
8	71.9 (0.1)	1.7 (0.0)	19.3 (0.0)
9	62.7 (0.1)	12.2 (0.2)	18.2 (0.4)

Figure 1: Dry matter yield (A) and cellulose recovery (B) after pretreatment at different glycerol concentrations
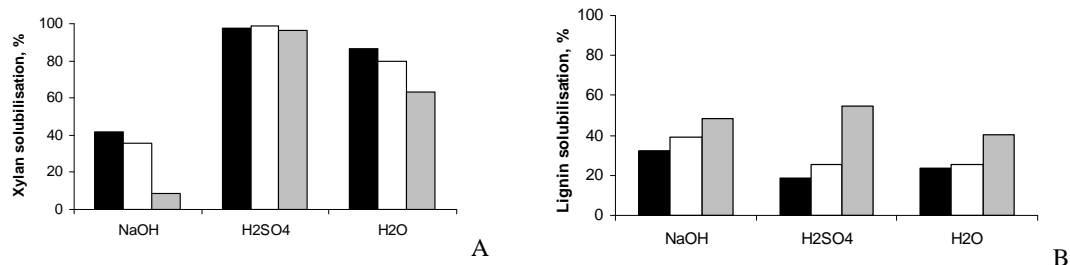
Figure 2: Solubilisation of xylan (A) and lignin (B) during pretreatment at different glycerol concentrations. Same symbols as in Figure 1

The high lignin solubilisation at high glycerol concentration agrees with literature results. Demirbas reported[12] a delignification degree above 90% for a 72% glycerol treatment. It should be remarked that the operational parameters (1% NaOH, 9 h) reported in the above-cited paper were considerably harsher than those used in the current work.

Pretreatment at different reaction times

To assess the effect of the reaction time on pretreatment effectiveness, bagasse was cooked with 60% glycerol for 1 to 4 hours (Experiments 4-6 and 10-18, Table 1). Generally, it was observed that the increase of the reaction time reduced the yield of pretreated solids (Fig. 3A), which is mainly

due to xylan solubilisation, seen as steadily increasing as the treatment proceeded (Fig. 4A). Around 60 and 93% of the initial xylan were solubilised after 4 h, respectively, in the alkaline and in the aqueous pretreatment. In the H_2SO_4-assisted pretreatment, xylan solubilisation also increased with time, although the relative increase was lower, since even in the shortest experiment, more than 98% xylan was already solubilised.

Worth mentioning is the lack of correspondence between xylose concentration in the pretreatment liquors and what was expected according to the xylan solubilisation pattern. Although xylan solubilisation increased, xylose concentration in the liquors decreased (Fig. 5), which might be a result of its degradation.
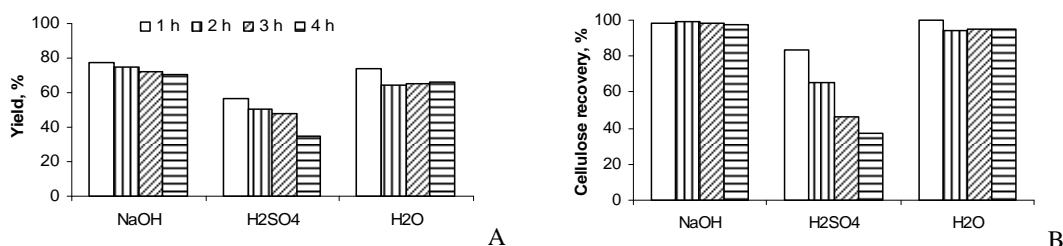

Figure 3: Dry matter yield (A) and cellulose recovery (B) in fibres after pretreatment at different reaction times
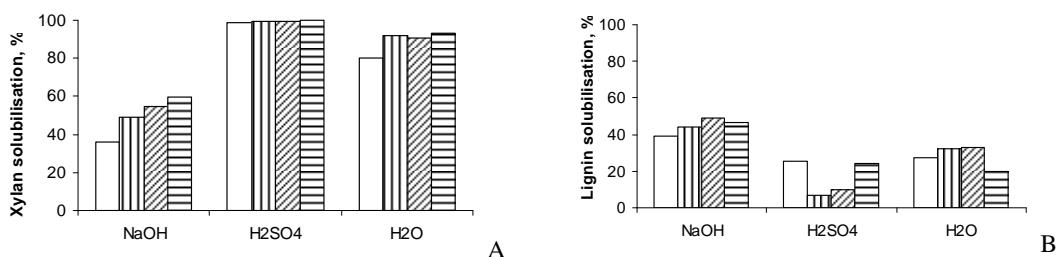

Figure 4: Solubilisation of xylan (A) and lignin (B) during pretreatment at different reaction times (right). Same symbols as in Figure 3
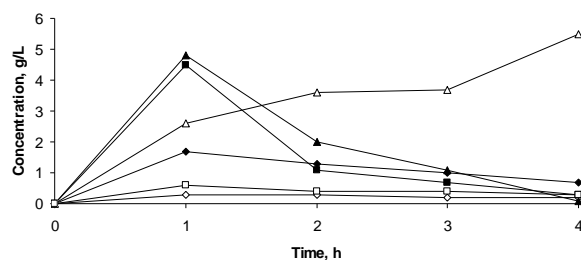
Figure 5: Concentration of xylose (filled symbols) and glucose (empty symbols) in filtrates obtained after glycerol pretreatment with NaOH (rhombs), H_2SO_4 (triangles) and without other chemicals (squares)
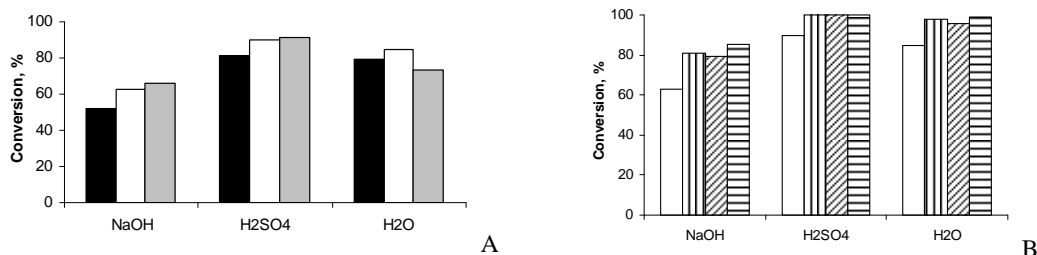

Figure 6: Enzymatic conversion (based on pretreated material) of cellulose in fibres resulting from pretreatments performed at different glycerol concentrations (A) and different reaction times (B). Same symbols as in Figures 1 and 3
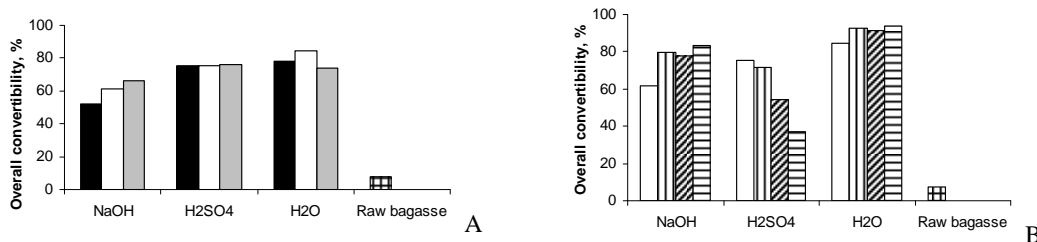

Figure 7: Overall enzymatic convertibility (based on raw material) of cellulose in fibres resulting from pretreatments at different glycerol concentrations (A) and different reaction times (B). Same symbols as in Figures 1 and 3
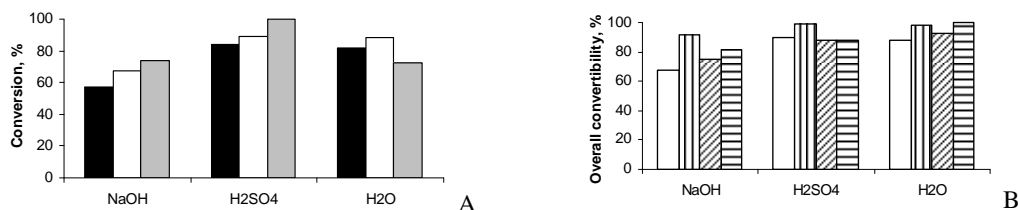

Figure 8: Enzymatic conversion of xylan remaining in pretreated material resulting from pretreatment at different glycerol concentrations (A) and different reaction times (B). Same symbols as in Figures 1 and 3

The sharpest decrease was observed in the H_2SO_4-assisted pretreatment, where the amount of detected xylose was the highest in the 1 hour experiment, and the lowest in the 4 hour one, which agrees with the known pattern of sugar degradation in high-temperature acidic media.[25]

The effect of the increased reaction time on cellulose and lignin was not as straightforward as on xylan. In the H_2SO_4-assisted pretreatment, a steady decrease of cellulose recovery in the cellulignin, down to 36.8% after 4 h (Fig. 3B), concomitantly with an increase of glucose concentration in the liquors from 2.6 to 5.5 g/L (Fig. 5), was observed as the reaction time increased. In the other pretreatments, cellulose was well-preserved, independently on the cooking time. On the other hand, the increase of the pretreatment time slightly improved lignin solubilisation in the NaOH-assisted process, while its effect on the delignification degree in the other two treatments was imprecise (Fig. 4B). These results agree with previous reports on alkaline glycerol treatments of *Ailantus altissima* and *Spruce orientalis* wood, regarding the increase of delignification with reaction time.[18]

However, the good cellulose recovery observed in the current work disagrees with the significant losses of cellulose previously detected in alkaline glycerol pulping of different wood species and agricultural residues.[12]

Enzymatic convertibility

The effectiveness of the glycerol pretreatment was revealed by the fact that enzymatic hydrolysis of cellulose in all pretreated materials was enhanced, comparatively with that observed for untreated bagasse.

The enzymatic convertibility of cellulose was calculated based on either pretreated material or raw bagasse. Enzymatic convertibility based on the pretreated material increased with glycerol concentration for alkaline and acidic pretreatments (Fig. 6A), and with pretreatment time for all pretreatments (Fig. 6B). The highest values were achieved in the H_2SO_4-assisted pretreatment, when convertibility was generally above 90%. However, since in the presence of sulphuric acid cellulose underwent considerable solubilisation during pretreatment, the overall convertibility, based on untreated bagasse, was only around 75% in the 1 hour experiment, dropping to 37% in the 4 hour one (Fig. 7).

The overall enzymatic convertibility was higher when bagasse was pretreated with glycerol alone (85-94%) than when it was assisted with either of the used chemicals. The obtained values are in good agreement with literature data, reporting around 90% enzymatic hydrolysis yields in aqueous industrial glycerol-pretreated wheat straw.[2] The increase in glycerol concentration led to increased overall convertibility for the alkaline pretreatment, whereas no clear effects were observed for the other pretreatments (Fig. 7A). Increasing the reaction time led to increases of overall convertibility for alkaline and aqueous pretreatments, whereas a sharp decrease was observed for the acidic one (Fig. 7B).

The xylan remaining in the fibres was partially hydrolysed during the assays of enzymatic convertibility of cellulose (Fig. 8), which can be explained by the xylanase activity of the used enzymatic preparation. This is an expected result, since it has been previously observed that bagasse xylan can be hydrolysed to some extent by

Trichoderma-derived cellulase preparations.[26] Furthermore, it is known that *Trichoderma* fungi are strong producers of xylanases, β-xylosidases and other hemicellulases,[27] and that Celluclast preparations have a remarkable β-xylosidase activity.[28]

CONCLUSIONS

Cellulose was almost totally recovered in the pretreated solids by aqueous and alkaline glycerol pretreatments, but it was remarkably hydrolysed in the H_2SO_4-assisted pretreatment.

Xylan solubilisation was maximal in the H_2SO_4-assisted pretreatment, independently of glycerol concentration and reaction time, whereas in the other pretreatments it decreased with the increase of glycerol concentration and increased with the length of the pretreatment.

Lignin solubilisation increased proportionally with glycerol concentration in the reaction mixture. At high concentrations, glycerol acts more selectively on lignin than on xylan. Selectivity was the highest in the NaOH-assisted pretreatment.

Glycerol pretreatment was effective in enhancing the enzymatic hydrolysis of cellulose. Although H_2SO_4 contributed to a high activation of fibres, and NaOH led to increased cellulose enzymatic conversion at high glycerol concentrations, the highest overall enzymatic convertibilities were achieved in the aqueous glycerol pretreatment.

REFERENCES
[1] M. Bender, *Bioresource Technol.*, **70**, 81 (1999).
[2] F. Sun and H. Chen, *Bioresource Technol.*, **99**, 5474 (2008).
[3] A. Behr, Protocols of the University of Dortmund, 2007, Available at http://www.uni-protokolle.de/nachrichten/id/131591/. Consulted on June 4, 2011.
[4] A. Hoogendoorn, T. Adriaans, J. M. N. van Kasteren and K. M. Jayaraj, Report No. 0656632-

R06, Ingenia Consultants & Engineers, Eindhoven, The Netherlands, 2007.

[5] A. Behr, J. Eilting, K. Irawadi, J. Leschinski and F. Lindner, *Chem. Today*, **26**, 32 (2008).

[6] S. Papanikolaou, S. Fakas, M. Fick, I. Chevalot, M. Galiotou-Panayotou, M. Komaitis, I. Marc and G. Aggelis, *Biomass Bioenerg.*, **32**, 60 (2008).

[7] S. Ashok, S. M. Raj, C. Rathnasing and S. Park, *Appl. Microbiol. Biot.*, **90**, 4 (2011).

[8] Y. Z. Xu, R. C. Wu, Z. M. Zheng and D. H. Liu, *World J. Microb. Biot.*, **27**, 6 (2011).

[9] Z. Lazar, E. Walczak and M. Robak, *Bioresource Technol.*, **102**, 6982 (2011).

[10] M. G. Vizoso Pinto, S. E. Pasteris and A. M. Strasser de Saad, *Food Microbiol.*, **1**, 111 (2004).

[11] T. Ito, Y. Nakashimada, K. Senba, T. Matsui and N. Nishio, *J. Biosci. Bioeng.*, **100**, 260 (2005).

[12] A. Demirbas, *Bioresource Technol.*, **63**, 179 (1998).

[13] J. Puls, in "Degradation of lignocellulosics in ruminants and in industrial processes", edited by J. M. van der Meer, B. A. Rijkens and M. P. Ferranti, Elsevier Applied Science Publishers Ltd., London, 1987, pp. 75-83.

[14] T. N. M. T. Ismail, H. A. Hassan, S. Hirose, Y. Taguchi, T. Hatakeyama and H. Hatakeyama, *Polym. Int.*, **59**, 181 (2010).

[15] F. Sun and H. Chen, *J. Chem. Technol. Biotechnol.*, **82**, 1039 (2007).

[16] C. A. Cardona, J. A. Quintero and I. C. Paz, *Bioresource Technol.*, **101**, 4754 (2010).

[17] G. J. M. Rocha, C. Martín, I. B. Soares, A. M. Souto-Maior, H. Baudel and C. A. M. Abreu, *Biomass Bioenerg.*, **35**, 663 (2011).

[18] M. M. Küçük, *Energ. Source. Part A*, **27**, 1245 (2005).

[19] E. Saberikhah, J. Mohammadi Rovshandeh and P. Rezayati-Charani, *Cellulose Chem. Technol.*, **45**, 67 (2011).

[20] W. J. J. Huijgen, A. T. Smit, J. H. Reith and H. den Uil, *J. Chem. Technol. Biot.*, DOI: 10.1002/jctb.2654 (2011).

[21] J. Puls, in "Bioconversion of forest and agricultural residues", edited by J. N. Saddler, CAB International, Wallingford, 1993, pp. 13-32.

[22] C. Martín, Y. López, Y. Plasencia and E. Hernández, *Chem. Biochem. Eng. Q.*, **20**, 443 (2006).

[23] C. Martín and A. B. Thomsen, *J. Chem. Technol. Biot.*, **82**, 174 (2007).

[24] R. Sanjuán, J. Anzaldo, J. Vargas, J. Turrado and R. Patt, *Holz Rohr Werkst.*, **59**, 447 (2001).

[25] D. Fengel und G. Wegener, "Wood Chemistry. Ultrastructure, Reactions", Walter de Gruyter, Berlin, 1989, pp. 274-276.

[26] C. Martín, H. Klinke and A. B. Thomsen, *Enzyme Microb. Techn.*, **40**, 426 (2007).

[27] A. O. Converse, in "Bioconversion of forest and agricultural residues", edited by J. N. Saddler, CAB International, Wallingford, 1993, pp. 93-106.

[28] A. S. Schmidt, M. Tenkanen, A. B. Thomsen and A. Woidemann, Hydrolysis of solubilised hemicelluloses derived from wet-oxidized wheat straw by a mixture of commercial fungal enzyme preparations, Risø-R-1042(EN), Risø National Laboratory, Denmark, 1998, 40 p.