Green Chemistry

Cite this: *Green Chem.*, 2011, **13**, 2764

www.rsc.org/greenchem

PAPER

High pressure pre-treatments promote higher rate and degree of enzymatic hydrolysis of cellulose

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Received 3rd May 2011, Accepted 30th June 2011
DOI: 10.1039/c1gc15500h

The effect of high pressure (HP) pre-treatments on the subsequent enzymatic hydrolysis of cellulose from bleached kraft *Eucalyptus globulus* pulp by cellulase from *Tricoderma viride* was evaluated. Pressure pre-treatments of 300 and 400 MPa during 5–45 min, lead to both an increased rate and degree of hydrolysis, reaching values ranging from 1.5- to 1.9-fold, quantified by the formation of reducing sugars. Both the pressure and time under pressure influenced the enzymatic hydrosability of the cellulosic pulps, with the former being more important. The results indicate that the pressure pre-treatments promoted an increased accessibility of cellulose towards cellulase in the cell wall. The results obtained open promising possibilities, to contribute to overcome conventional limitations of enzymatic cellulose hydrolysis for the production of fermentable glucose, for the production of second generation bioethanol and chemicals by enhancement of both rate and yield of hydrolysis. The results are also of interest for the preparation of "pressure engineered" celullose with incremented tailored hydrolysis patterns.

Introduction

The inevitable reduction of dependence from fossil fuels for energy and chemicals production has increased attention to the use of renewable biomaterials for these processes.[1–3] In this context, significant efforts are now being made to develop reliable and economically viable technologies for the conversion of plant resources into bioethanol and chemicals synthesis, *via* bioprocessing of fermentable sugars.[4–7]

Plant cell walls are composed mainly by three major structural polymers: cellulose, hemicelluloses and lignin. Cellulose, the most abundant structural polysaccharide composed by repeated D-glucopyranose units linked by β-(1→4)-glycosidic bonds, is a very promising source of fermentable glucose.[8,9] In plant cell walls, cellulose molecules aggregate into elementary fibrils (EF) which, in turn, are assembled into microfibrils (MF). MF are embedded into a matrix of lignin and hemicelluloses.[8,10] This structural hierarchy hinders both chemical and enzymatic hydrolysis of cellulose, being the last one particularly difficult. Being an amorphous-crystalline polymer, cellulose provides additional difficulties to hydrolysis in terms of accessibility into crystalline domains, by the tight packing of cellulose chains.[10] A poor efficiency of enzymatic hydrolysis of lignocellulosic materials represents an obstacle for a successful use of these naturally abundant renewable sources for production of second-generation fuels and chemicals.[11–13] For this reason, numerous pre-treatment techniques are being developed in order to improve cellulose accessibility and thus hydrolysis efficiency (rate and yield).[14–18] The main approaches include application of physical methods to disintegrate plant tissues and chemical/biochemical treatments to eliminate concomitant biopolymers (mainly lignin and hemicelluloses) hindering cellulose accessibility.[19] However, improvements of cellulose hydrolysis promoted by these methods have been limited.[13,20]

High pressure (HP) is now an established technology for food preservation,[21] with potential also to modify the properties of macromolecules[22] and influence physiological processes.[23] Recently it was reported that HP treatments of cellulosic pulps are a promising tool for non-degradative modification of cellulose fibres properties.[24] In this work it was shown that HP (400 MPa, ~4000 atm) causes the rearrangement of EF in cellulosic fibres, in such a way that those became less aggregated and more hydrated (containing increased amounts of strongly bound water). This HP treatment caused also a significant improvement of cellulose accessibility towards hydrolysis with diluted sulphuric acid. In another recent work, it was shown that hydrolysis of carboxymethyl cellulose, used as a case study, in the presence of the ionic liquid [bmim]Cl could be improved by carrying out the hydrolysis under HP.[25]

Based on these results and following the same rational strategy, the effect of HP, as a pre-treatment, on the subsequent enzymatic hydrolysis of cellulosic fibres by cellulase was evaluated in the present work, aiming to improve cellulose rate and degree of hydrolysis.

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Materials and methods

Preparation and chemical characterization of cellulosic pulp

Cellulosic *Eucalyptus globulus* bleached kraft industrial pulp supplied by Portucel (Portugal) was used in this work. Pulp was swollen in water during 48 h under moderate agitation, filtered off and dried on air at 20 °C to humidity around 8%.

The kraft pulp was characterized for ash, extractives, residual lignin and sugar composition of its constituent polysaccharides. Ash content was determined by complete incineration of pulp at 525 ± 25 °C during 3 h in a muffle furnace according to NP 3192 norm. Extractives were removed by Soxhlet extractions during 4 h with acetone (TAPPI method T204 om-88). Klason lignin was determined according to Tappi standard T 222 om-88. Neutral sugars analysis of pulp was carried out after its two-step Saeman hydrolysis by GC-FID as alditol acetates.[20] All analyses were done in duplicate.

High pressure treatment of cellulosic pulp

Before the HP treatments, air-dried pulp was swollen in a 0.05 M sodium acetate buffer solution (pH 5) during 24 h under agitation. A pulp suspension containing 0.040 g of pulp (1.1 mg of pulp per mL) in the same buffer was placed into flexible plastic flasks that were in turn introduced in plastic bags and heat-sealed under vacuum. HP treatments were carried out in a High Pressure U33 device (Institute of High Pressure Physics, Poland), equipped with a high pressure vessel of *ca.* 100 cm^3 capacity, at 300 or 400 MPa during 5, 15 or 45 min, at room temperature (*ca.* 20 °C). The pressure transmitting medium used was propylene glycol : water, 1 : 1 (v/v).

Enzymatic hydrolysis

A commercial endo-cellulase (E.C.# 3.2.1.4) from *Tricoderma viride* (C9422, 9 U mg^{-1}, from Sigma–Aldrich Co., St. Louis, MO) was used in the hydrolysis experiments. For comparison reasons, HP-treated and non-treated pulps (both swollen in 0.05 M sodium acetate) were hydrolyzed. The conditions of enzymatic hydrolysis under agitation were selected as follows (after preliminary experiments to identify enzyme/pulp ratios that showed adequate rate of hydrolysis): pulp amount in 0.05 M sodium acetate (pH 5) solution – 0.6% (wt.); cellulase load – 9 U mg^{-1} pulp; temperature – 40 °C; total reaction volume – 8.0 mL. The reaction was monitored by determination of reducing sugars (RS) release along the hydrolysis, which were quantified with 3,5-dinitrosalicylic acid (DNS) as described previously,[26] using glucose as calibration standard.

Optical microscopic analysis

Selected samples of suspensions of hydrolysed pulps, with and without HP treatment, were analysed on an optical microscope OLYMPUS BX51, equipped with a digital camera OLYMPUS C4040 (4.1 megapixel resolution), to verify possible changes on cellulose fibres, resulting from hydrolysis after the HP treatments.

Table 1 Chemical composition of eucalypt pulp

	Abundance (%)	Usual values (%)[27]
Ash	0.12	0.10–0.30
Extractives in acetone	0.18	0.15–0.23
Residual lignin	0.20	0.10–0.30
Neutral polysaccharides:		
Glucan	86.0	80.4–86.0
Xylan	13.5	13.0–18.5
Others	0.5	0.3–1.5

Results and discussion

Chemical composition analysis of the pulp used revealed values in the range usually obtained for *Eucalyptus globulus* bleached kraft industrial pulps (Table 1).

As can be seen in Fig. 1 (left *Y*-axis), HP pre-treatments caused a faster formation of RS, when compared to non-treated pulp, up to 1.9-fold. This effect was dependent on both applied pressure and the exposure time, though the former was more significant. This reflects on the relative increase of hydrolysis rate or enzymatic activity caused by HP (Fig. 1, right *Y*-axis): the effect observed for the pulp treated at 300 MPa for 45 min was similar to that of pulp treated at 400 MPa for 5 min only. Accordingly, the importance of duration of the HP

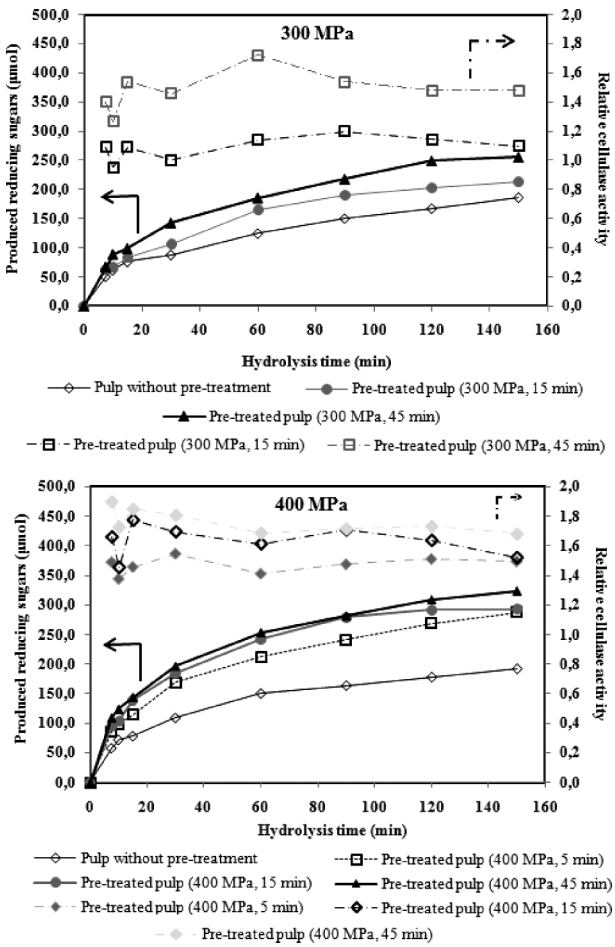

Fig. 1 Production of reducing sugars (left *Y*-axis) and cellulase relative activities (right *Y*-axis) during the enzymatic hydrolysis of HP-treated and non-treated pulps.

treatment became less pronounced when HP was increased from 300 to 400 MPa (the amount of released RS and the relative enzymatic activity was practically the same after 15 and 45 min of pulp treatment at 400 MPa). The observed higher amounts of RS formation for the HP-treated pulps, throughout the whole hydrolysis reaction, indicates a facilitated access (both easier and higher access) of the enzyme to the glycosidic bonds to cleave.

Easier access should be more determinant at the beginning of the hydrolysis, becoming less significant as hydrolysis proceeds, and would result in higher effects of HP for shorter hydrolysis times. In fact, the increment of RS formation caused by HP tended to be slightly higher for shorter hydrolysis times, with maximum values being verified for 45 min at 300 and 400 MPa, respectively 1.4- and 1.9-fold, based on increment of initial velocity (v_0) of hydrolysis. Higher access of the enzyme to cellulose, indicates that the enzyme has access to glycosidic bonds that are not accessible in the non-treated pulps, allowing a more extensive hydrolysis, evidenced by the higher RS formation, when the enzymatic hydrolysis is approaching the plateau, and no more hydrolysis occurs. At the end of the experiments (150 min of hydrolysis), HP-treated pulps show higher degrees of hydrolysis (increments of 1.2- and 1.5-fold for 300 MPa and 1.5- to 1.7-fold for 400 MPa).

A deeper quantitative analysis, reveals that plotting the relative formation of RS (RS formed for HP-treated/non-treated pulp), for 400 MPa (for which 5, 15, and 45 min of processing were studied), *versus* the HP treatment time, results in straightlines for each hydrolysis time, thus indicating a kinetic effect of HP treatment time on "hydrosability" of cellulose. Moreover, the slopes obtained decrease linearly up to 30 min of hydrolysis and then reach a plateau (Fig. 2). This linear correlation supports the assumptions of the hypothesis stated above: for the initial hydrolysis times, HP cause both an easier and increased access, with the former becoming less important for the longer hydrolysis times (as quantified by the linear decrease shown in Fig. 2), till becoming no significant (when the plateau occurs).

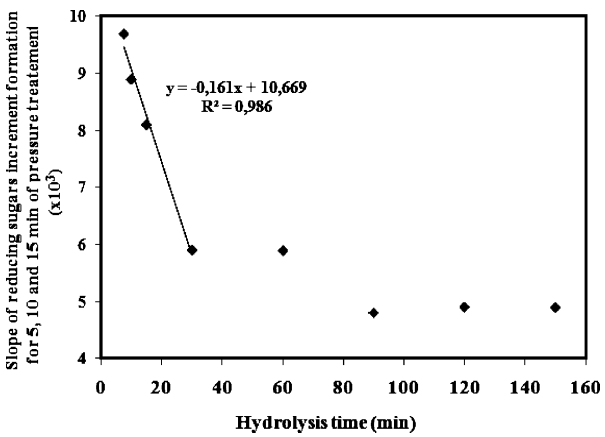

Fig. 2 Plot of the slopes obtained by linear regression of the relative increment of RS formation for 400 MPa (5, 15, and 45 min of treatment) for each hydrolysis time, as a function of the hydrolysis time.

On a physical molecular basis, the results observed are likely to be related with the forced hydration of pulps caused by HP, leading to increased amounts of strongly bound water linked to cellulose and swelling of the fibres, as previously reported.[28] This hydration and swelling effect should "open" ways to the enzyme to the cellulosic fibres, thus allowing easier and more extensive access to the glycosidic bonds to cleave.

The observed enhanced rate (enzyme activity) and degree of hydrolysis may be also related with HP contributing to overcome the difficulty posed by the architectural topochemistry of cellulosic fibres to cellulose access and hydrolysis. HP may allow faster hydrolysis in rather accessible external layers of the fibre (easier access during the first minutes of the reaction) and promoting also augmented cellulase penetration into the fibre bulk depth, where MF are less accessible physically, covered with xylan bounded to small amounts of residual lignin (*ca.* 0.2% w/w), thus promoting access to previously inaccessible regions (more extensive hydrolysis).

The aforementioned results evidenced that HP treatment of cellulosic fibres promoted their accessibility towards cellulase, improving both the rate and the extent of enzymatic hydrolysis. These effects may be controlled by appropriate selection of HP treatment conditions: the pressure and the exposure time.

The increased accessibility of eucalypt kraft pulp towards cellulase may be explained by the changes occurred in the supramolecular structure of cellulose fibres during the hyperbaric treatment. As suggested previously,[19] under HP part of neighbor EFs, the surfaces of which are free of concomitant hemicelluloses and appropriately oriented, suffer structural rearrangements leading to the junction of adjacent cellulose crystallites (cocrystallization). Simultaneously, other EFs suffer increased disaggregation due to forced hydration of inaccessible internal surfaces thus promoting the increase of interfibrilar spaces. The aggregation of MF is also decreased remarkably by HP treatments. These features are schematically depicted in Fig. 3.

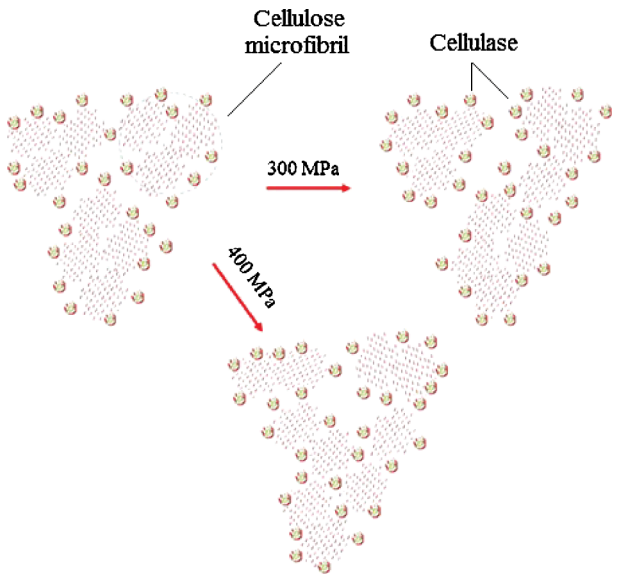

Fig. 3 Schematic representation of cellulose microfibrils (MFs) behavior when submitted to HP treatments.

What might be occurring is that at a pressure of 300 MPa the disaggregation of EFs is less accentuated than at 400 MPa

thus promoting a lower cellulase penetration into inaccessible areas. It may be also proposed that HP treatment favours more uniform hydrolysis of cellulosic fibres due to better accessibility to both amorphous and crystalline domains. This assumption is supported by analysis of hydrolysed (120 min) HP-treated and non-treated fibres in suspension by optic microscopy (Fig. 4). After the hydrolysis, non-treated pulp showed larger and less swollen fibre particles than pulp HP-treated at 400 MPa during 15 min, that revealed more swollen fibre particles in clouds of cellulose microcrystals. These observations support the hypothesis that the pressure pre-treatments increase the accessibility of cellulase to cellulose catalytic action, thus enhancing the enzymatic activity.

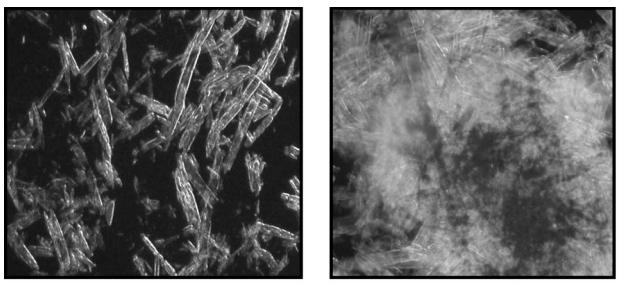

Fig. 4 Optic microscope images (400× zoom) of HP pre-treated (400 MPa, 15 min) and non-treated hydrolyzed cellulosic fibres of *E. globulus* kraft pulps.

Conclusions

The results of this work reveal that high pressure pre-treatments (300 and 400 MPa) are an effective tool to enhance hydrolyis of cellulosic fibres of bleached kraft *Eucalyptus globulus* pulp, with an endo-cellulase from *Tricoderma viride*. The fibre treatment at high pressure promoted the increase of cellulose accessibility to the enzyme catalytic action. Both rate and degree of hydrolysis augmented in the HP-treated cellulosic pulps, reaching a value up to 1.7-fold higher degree of hydrolysis, quantified by the formation of reducing sugars. The results indicate that high pressure treatments are very promising to promote more easy and extensive cellulose enzymatic hydrolysis, to produce sugars for second generation bioethanol and chemicals production. The rate and the degree of enzymatic hydrolysis may be controlled by selection of appropriate HP treatment conditions (primarily pressure and exposure time), thus allowing the production of "pressure engineered" cellulosic fibres, with respect to the desired hydrolysis extent and profile, with possible different properties for new applications. Overall, HP treatment is a very promising technology to facilitate cellulose hydrolysis.

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