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Cellulase stability, adsorption/desorption profiles and recycling during successive cycles of hydrolysis and fermentation of wheat straw

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HIGHLIGHTS

- Characterization of the enzyme distribution between the liquid and solid fractions.
- More efficient hydrolysis leads to higher recovery in the liquid fraction.
- Enzyme recycling critically depends on thermostability.
- Appropriated choice of the process conditions may lead to efficient enzyme recycling.

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ABSTRACT

The potential of enzymes recycling after hydrolysis and fermentation of wheat straw under a variety of conditions was investigated, monitoring the activity of the enzymes in the solid and liquid fractions, using low molecular weight substrates. A significant amount of active enzymes could be recovered by recycling the liquid phase. In the early stage of the process, enzyme adsorb to the substrate, then gradually returning to the solution as the saccharification proceeds. At 50 °C, normally regarded as an acceptable operational temperature for saccharification, the enzymes (Celluclast) significantly undergo thermal deactivation. The hydrolysis yield and enzyme recycling efficiency in consecutive recycling rounds can be increased by using high enzyme loadings and moderate temperatures. Indeed, the amount of enzymes in the liquid phase increased with its thermostability and hydrolytic efficiency. This study contributes towards developing effective enzymes recycling strategies and helping to reduce the enzyme costs on bioethanol production.

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1. Introduction

Bioethanol derived from the bioconversion of lignocellulosic feedstocks continues to attract global interest as an alternative to current petroleum-based fuels. However, considerable technical improvements are still needed before efficient and economically feasible lignocellulosic biomass-based ethanol processes can be commercialized. One of the major limitations of this process is the consistently high cost of the enzymes involved in the conversion of cellulose into fermentable sugars (Lynd et al., 2008; Klein-Marcusschamer et al., 2012).

Several strategies, such as increasing substrate reactivity through lignin removal or modification (Zhu et al., 2009a; Kumar et al., 2011) and enzyme recycling have been investigated (Otter et al., 1984, 1989; Tu et al., 2007a,b, 2009; Zhu et al., 2009b; Wu et al., 2010; Qi et al., 2011; Rodrigues et al., 2012; Lindedam et al., 2013; Seo et al., 2011). After enzymatic hydrolysis, cellulases can either remain bound to the residual biomass (solid fraction) or free in the supernatant (liquid fraction) (Tu et al., 2009; Yang et al., 2010; Pribowo et al., 2012; Lindedam et al., 2013). Therefore, the studies on the cellulases adsorption, desorption, and re-adsorption are important to provide fundamental understanding regarding the potential of cellulase recycling.

Two overall complementary strategies to recover cellulases may be conceived, one regarding the fraction of enzyme present in the liquid phase, the other the solid bound fraction. In this work, we focus mainly on the first approach. Free cellulases in bulk solution may be recovered by promoting its readsorption on fresh substrate, which may include or not an ultrafiltration step (Lindedam et al., 2013; Qi et al., 2011; Lee et al., 1995; Knutsen and Davis, 2004). Regarding the solid bound fraction, recycling the residual lignin with the adsorbed enzyme is an attractive approach, given its simplicity. However, the solid lignin residue increases with the number of recycling rounds, adversely impacting the hydrolysis of fresh substrate (Girard and Converse, 1993; Lee et al., 1995). Enzymes bound to the solid residue may also be recovered by using

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agents such as surfactants, alkali, urea, glycerol, polyethylene glycol and buffers of different pH's (Rad and Yazdanparast, 1998; Otter et al., 1989; Rodrigues et al., 2012; Zhu et al., 2009b; Desphande and Erikson, 1984; Sipos et al., 2010; Wang et al., 2012).

It is important to recognize that recycling enzymes, an highly desirable goal, is possible only as long as they are stable through several cycles (Tengborg et al., 2001; Pribowo et al., 2012; Rodrigues et al., 2012; Chylenski et al., 2012). It has been observed that along hydrolysis and fermentation enzymes loose activity, an effect critically dependent on temperature (Rodrigues et al., 2012; Chylenski et al., 2012; Gunjikar et al., 2001; Ye et al., 2012). In previous work we observed that incubation for 1 week at 50 °C reduces the activity of Cel7A by 62.5% (Rodrigues et al., 2012). Therefore, the use of thermostable enzymes – or operational conditions not compromising the enzymes stability – offer potential benefits in the hydrolysis of lignocellulosic substrates: enhanced stability allows for improved hydrolysis and increased flexibility with respect to process configurations, all leading to improvement of the overall economy of the process and increasing the potential for enzyme recycling.

This study describes the adsorption and activity profiles of specific enzymes present in Celluclast (Cel7A and Cel7B and β-glucosidase) during consecutive stages of hydrolysis and fermentation of pretreated wheat straw, using different hydrolysis temperature and enzyme loadings. In particular, the partition of enzyme between the solid and liquid fraction is analysed by monitoring the activity in each fraction by using low molecular weight substrates. Furthermore on basis of these results the second aim of this work was to determine whether by recycling the liquid fraction a significant amount of enzyme could be reused, and therefore increase overall product yields or decrease the amount of required enzyme needed to reach a given level of conversion.

2. Methods

2.1. Enzymes and substrate

Enzymatic hydrolysis was carried out using enzyme preparations, Celluclast 1.5 FG L combined with β-glucosidase (Novozyme 188) (all from Novozymes A/S, Bagsvaerd, Denmark). Wheat straw was processed by hydrothermal pretreatment at the Inbicon pilot plant (Petersen et al., 2009) and stored at 4 °C. The pretreated wheat straw, used as substrate in the experiments, contained 52.82% cellulose, 2.47% xylan, 39.03% lignin Klason and 3% ash, as determined by acid hydrolysis (Section 2.4.3).

2.2. Hydrolysis, fermentation and desorption

The hydrolysis and fermentation were conducted under different conditions of temperature and enzyme loading, as described below.

Hydrolysis and fermentation were performed in 500 mL Erlenmeyer flasks in an incubator shaker (Unimax 1010 Heidolph) with a rotational mixing at 160 rpm. Enzymatic hydrolysis was carried out on 150 mL of 0.1 M sodium acetate buffer (NaAc, Sigma–Aldrich, 32318) at pH 4.8, using a concentration of biomass (wheat straw) of 5% (w/v) on a dry weight basis. Celluclast was added at two different loadings, 20 and 40 Filter Paper Units (FPU)/g cellulose, supplemented with β-glucosidase to 40 IU/g cellulose.

Hydrolysis of the biomass was performed at 37 °C or 50 °C for 48 h. Afterwards, the fermentation flasks were cooled down to room temperature and inoculated with *Saccharomyces cerevisiae* CEN PK 113 wild type with an initial optical density ($O.D._{600}$) = 0.1. Other nutrients required for the fermentation stage were added to a final concentration of 1% (w/v) yeast extract and 2% (w/v) peptone. The flasks were then incubated at 37 °C for 120 h, 160 rpm. All the experiments were carried out under sterile conditions. Samples were taken at the beginning of the assay and every 24 h, up to 168 h, centrifuged at 4480*g* for 12 min (microcentrifuge Sigma, model 113) and the supernatant was analysed for ethanol and sugars by HPLC (Section 2.4.4.). The enzymatic activities associated to liquid and solid fraction as well as the total activity were measured along the process, every 24 h (Section 2.4.1), in order to evaluate the adsorption/desorption profiles along hydrolysis/fermentation and the thermo stability of Celluclast.

Several consecutive rounds of hydrolysis/fermentation were performed using the recycled enzyme (Section 2.3). At the end of each round, the solid residue was separated from the liquid fraction by centrifugation at 13,131*g* for 30 min (Sigma 4K15), the supernatant was collected for additional treatment and subsequent recycling (described below) and the final solid residue composition was analysed for estimation of the biomass degree of conversion (Section 2.4.3).

2.3. Cellulase recycling after each round of hydrolysis and fermentation of wheat straw

The recovery of cellulases after fermentation was performed by filtering the liquid phase through a 0.22 μm Polyethersulfone (PES) Membrane ACROVAC (PALLAVFP02S) followed by concentration and buffer exchanging with fresh 0.1 M NaAc buffer, pH 4.8 in a tangential ultrafiltration system Pellicon XL membrane with a 10 kDa cut-off PES membrane (Millipore, Billerica, MA, USA). The recovered cellulase, buffer and nutrients were then added to fresh wheat straw substrate, at 5% (w/v) on a dry weight basis to carry out the next round of hydrolysis and fermentation. At each new recycling round, fresh enzyme was added, corresponding to 20% of initial load of each enzyme, i.e. 4 FPU Celluclast: 8 IU β-glucosidase/g cellulose and 8 FPU Celluclast: 8 IU β-glucosidase/g cellulose, in order to compensate the enzyme lost (either due to deactivation or some loss of material in the manipulation of the samples, namely in the ultrafiltration stage). The conditions (time, temperature, mixing) of hydrolysis and fermentation, time point for sample collected and analysis of two consecutive rounds of enzyme recycling (R1) and (R2) were the same as in the first stage of fermentation (R0).

2.4. Analytical methods

2.4.1. Enzyme activity measurements

Cel7A, Cel7B and β-glucosidase activities were measured by fluorescence spectroscopy using a Biotech Synergy HT Elisa plate reader and 4-methylumbelliferyl-β-D-cellobioside (MUC, Sigma–Aldrich, M6018), 4-methylumbelliferyl-β-D-lactopyranoside (MULac, Sigma–Aldrich, M2405) and 4-methylumbelliferyl-β-D-glucopyranoside (MUGlc, Sigma–Aldrich, M3633) as substrates, respectively. Upon hydrolysis by Cel7A, Cel7B and β-glucosidase, the substrates release free 4-methylumbelliferone (MU, Sigma–Aldrich, M1508) resulting in a shift of the fluorescence spectra (excitation maximum/fluorescence maximum), which was quantified for excitation and emission wavelengths of 360 and 460 nm respectively.

The Cel7A, Cel7B and β-glucosidase activities were measured by adjusting the protocol published by Bailey and Tähtiharju (2003). In these assays, 400 μL of 1 mM MUC, MULac or MUGlc solutions (in 0.1 M NaAc buffer, pH 4.8) were added to 50 μL of the test samples (dilutions in NaAc buffer); the mixture was vortexed and incubated at 50 °C for 15 min. The reaction was then stopped by addition of 550 μL 1.0 M Na_2CO_3 buffer (Panreac, 131647.1211) (for Cel7A and β-glucosidase activity

measurements) or 500 μL 1.0 M Na_2CO_3 buffer (Panreac, 131647.1211) (for Cel7B activity measurements) and measured on a black bottom 96-well UV fluorescence microplate. Since Cel7A, Cel7B and β-Glucosidase – all hydrolyse the MULac substrate, the Cel7B activity was measured adding 50 μL of a mixture containing 1.0 M glucose and 50 mM cellobiose to the chromophoric substrate MULac, in order to inhibit the Cel7A and β-glucosidase activities (van Tilbeurgh et al., 1982).

A 1 mM stock solution of MU was diluted from 0.0001 mM to 0.02 mM and used to prepare a standard calibration curve by plotting MU concentration (mM) versus relative fluorescence units (RFU). The amounts of MUC, MULac and MUGlc hydrolyzed (mM) were converted to enzyme activity units, (IU/mL), defined as the amount of enzyme that catalyzes the transformation of one micromole of substrate per minute under specified conditions. The amount of MUC, MULac or MUGlc hydrolyzed was determined using the calibration curves obtained. All assays were performed in triplicate.

2.4.2. Activity on filter paper fibers – FPase assay

The filter paper activity of the liquid fractions collected at the beginning and at the end of the process (before ultrafiltration) was measured.

The Cellulase activity was expressed in FPU in accordance with the standard analytical methods established by the National Renewable Energy Laboratory (Adney and Baker, 1996). One unit of filter paper cellulase activity (FPU) was defined as the amount of enzyme which produces 2.0 mg of reducing sugar from 50 mg of filter paper within 1 h. The experiment was carried out in a reaction mixture containing 0.5 mL of diluted samples enzyme solution, 1.0 mL of 0.1 M NaAc buffer (pH 4.8), and 50 mg of a 1 × 6 cm strip of a Whatman No. 1 filter paper. The reaction solution was incubated at 50 °C for 1 h. Then the concentration of the released reducing sugar was measured using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959).

2.4.3. Analysis of the composition of the solid residue

The solid residue obtained after hydrolysis and fermentation was dried at 37 °C to constant weight. Aliquots from the homogenized residue lot were subjected to moisture determination and quantitative acid hydrolysis with 5 mL of 72% (w/w) sulphuric acid for 1 h at 30 °C, with constant stirring, followed adding water until 148.67 g and autoclaved at 121 °C for 1 h. Thereafter, the solid residue from hydrolysis process was recovered by filtration with crisol Gooch n° 3 and drying at 105 °C to constant weight; this residue was classified as Klason lignin (Browning, 1967). The monosaccharides in the liquid fraction were analysed by HPLC (Section 2.4.4).

2.4.4. Sugar and ethanol analysis

All liquid samples taken from the hydrolysis and fermentation were filtered through a 0.2 μm (PES membrane, Ø 25 mm, VWR, 514-0072) and analysed for cellobiose, glucose and ethanol by HPLC. Chromatographic separation was performed using a Metacarb 87 H column (300 × 7.8 mm, Varian, USA) under the following conditions: mobile phase 0.005 M H_2SO_4, flow rate 0.7 mL/min, and column temperature 60 °C. The volume injected was 20 μL. The concentration of monosaccharides and ethanol were determined based on calibration curves of these pure compounds.

2.5. Calculations

2.5.1. Glucose yield

The glucose yield was calculated according to the NREL standard procedure (Down and McMillan, 2001):

$$\%\text{Yield} = \frac{[\text{Glucose}] + 1.053 \times [\text{Cellobiose}]}{1.111 \times f \times [\text{Biomass}]} \times 100\% \quad (1)$$

where [Glucose] is the residual glucose concentration (g/L). [Cellobiose] is the residual cellobiose concentration (g/L). [Biomass] is the dry biomass weight concentration at the beginning of the hydrolysis step (g/L); f is the cellulose fraction of dry biomass (g/g).

2.6. Statistical analysis

The statistical analyses were performed using GraphPad Prism version 5 for Windows, GraphPad Software, San Diego, California, USA.

3. Results and discussion

3.1. Enzyme stability and distribution of enzymes in the liquid and solid fractions

The thermal stability of cellulases (Cel7A and Cel7B) and β-glucosidase under the operational conditions was assessed, as this is of paramount importance in any enzyme recycling strategy. All three enzymes proved to be stable at 37 °C ("total enzyme activity" data on Fig. 1A and C), as the activity observed is constant throughout the process, up to 168 h, for each of the recycling rounds. However, when the hydrolysis was conducted at 50 °C, a significant reduction of enzymatic activity was observed in all cases ("total enzyme activity" data on Fig. 1B and D), particularly in the first 24 h, but also steadily throughout the entire process. Interestingly, the loss of enzyme activity was in every case more pronounced in the initial round (R0). A plausible explanation is thermal denaturation, deactivation by shear forces or contact with the air–liquid interphase. Cel7A and Cel7B seems to be slightly less stable than β-glucosidase, as the fraction of total activity reduction is more expressive in this case ("total enzyme activity" data on Fig. 1B and D).We have measured the activity of the different enzymes in the solid and liquid fractions along the process in order to analyse the effect of temperature and enzyme concentration on the distribution between the phases of the heterogeneous system and to define a suitable enzyme recycling strategy. In general, as expected and in accordance with many studies (Tu et al., 2009; Qi et al., 2011; Pribowo et al., 2012; Yang et al., 2010), we observed the adsorption of Cel7A and Cel7B, which was more pronounced at the initial phase of hydrolysis; at 24 h, more enzyme activity is adsorbed than in solution, especially in the case a lower load of enzyme was used. Increasing the enzyme loading from 20 FPU to 40 FPU resulted in a reduction of the fraction of enzyme activity adsorbed after fermentation, likely because of the higher conversion degree and saturation of the substrate surface (Fig. 1A–D). After the initial 24 h, enzymes desorbed continuously throughout the process, an effect more evident in the R0 round for the lower temperature. Therefore, at the end of fermentation, part of the active Cel7A and Cel7B remained attached to the final residue (Fig. 1A–D). Overall, the portion of enzyme adsorbed after 168 h seemed to increase as the cellulose conversion degree decreased, this being more evident in cases where the original enzyme loading was lower (Figs. 1 and 2). For example, under the conditions corresponding to Figs. 1A and 2A (20 FPU, hydrolysis at 37 °C) the conversion degree after 48 h dropped from 80% in the initial round (R0) to 50.2% and 31% in the following ones (R1 and R2). The desorption of enzyme was quite high during the initial round (70%), but not so expressive in the next ones (58% R1 and 49% R2), both for Cel7A and Cel7B. A similar trend was observed under the other conditions (Figs. 1B–D and 2B–D). Noteworthy, under the most effective conditions (40 FPU, hydrolysis at 37 °C, Fig. 2C), a high conversion degree was reached after 48 h in the consecutive

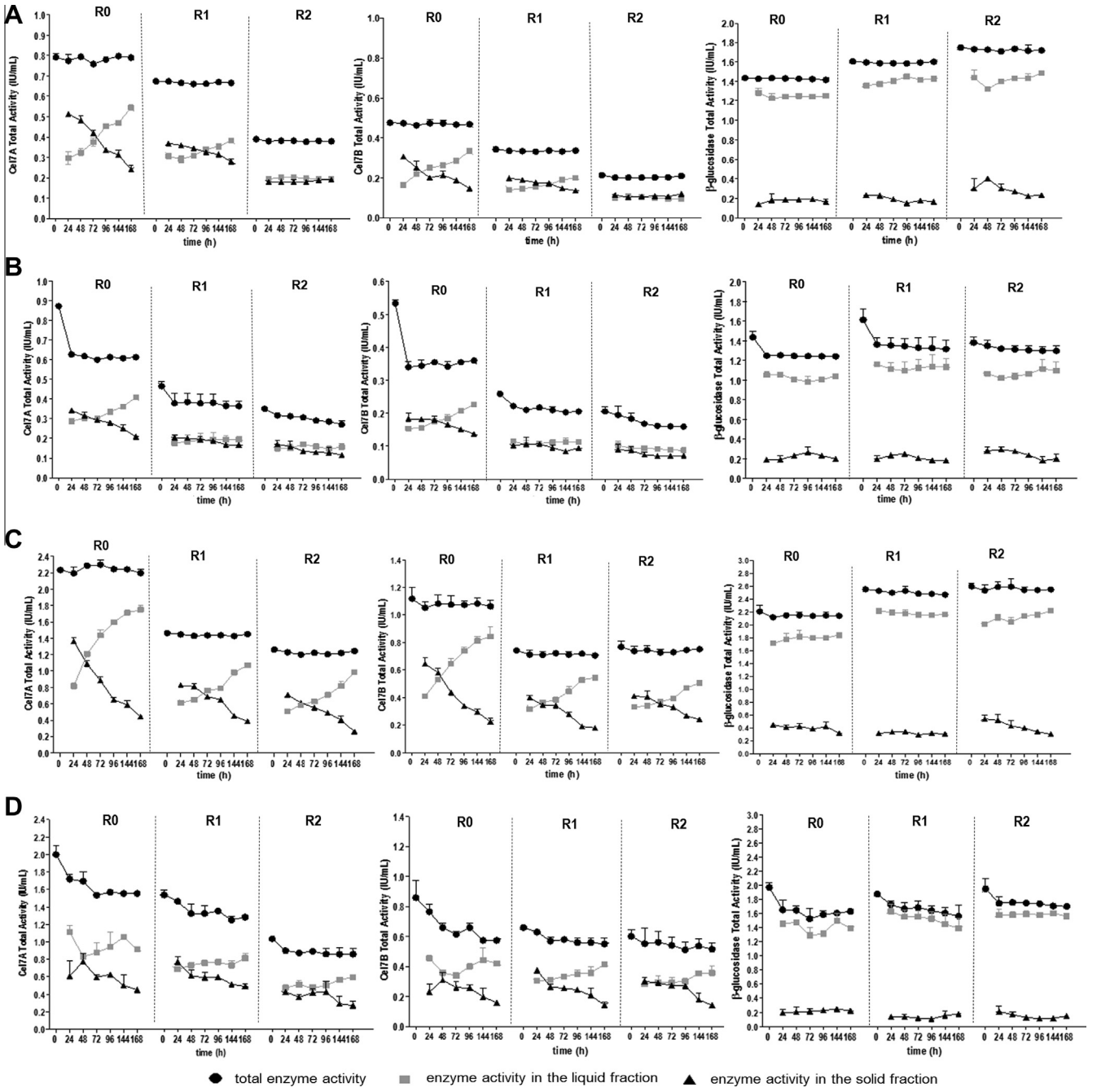

Fig. 1. Determination of Cel7A, Cel7B and β-glucosidase activities in solid and liquid fraction at different conditions of wheat straw hydrolysis and fermentation. (A) 20 FPU/g cellulose, hydrolysis at 37 °C, (B) 20 FPU/g cellulose, hydrolysis at 50 °C, (C) 40 FPU/g cellulose, hydrolysis at 37 °C, (D) 40 FPU/g cellulose, hydrolysis at 50 °C. R0, R1 and R2 refer, respectively, to the initial process and to the first and second rounds of enzyme recycling. Reported values are average of duplicates, error bars represent + standard deviation.

rounds (respectively 100%, 89% and 81%, for R0, R1 and R2), and in this case both Cel7A and Cel7B returned to the soluble phase after 168 h in higher amounts, in each round. It is remarkable that using 37 °C during hydrolysis results in a residual total activity at the end of R2 of about 1.2 and 0.75 IU/mL (Fig. 1C, total enzyme activity), respectively for Cel7A and Cel7B, higher than the observed activities for the case where a temperature of 50 °C has been used (0.86 and 0.52 IU/mL – Fig. 1D). This is certainly due to the thermal deactivation of enzymes, which is clearly not stable enough at 50 °C. Another result that highlights the critical relevance of the temperature in the overall yield obtained according to the time course of the reaction: in the initial round (R0), although after the first 24 h the degradation of the lignocellulosic material was more efficient when using higher temperatures (67% and 87% at 50 °C, 20 and 40 FPU, respectively; 60% and 75% at 37 °C, 20 and 40 FPU, respectively), after 48 h more efficient conversion rates were achieved using a lower temperature (77% and 97% at 50 °C, 20 and 40 FPU, respectively; 80% and 100% at 37 °C, and 20 and 40 FPU, respectively) (Fig. 2A–D R0). The significance of each results was examined using analysis of variance (ANOVA). For both enzyme loadings it was observed that the differences observed after 24 h, comparing the hydrolysis yield at 37 °C, was indeed significant ($p < 0.01$). At the end of 48 h of hydrolysis, the difference between two temperatures used is still significant, with $p < 0.05$. As we have shown in previous work, cellulases have lower binding affinity to lignin as compared to pure cellulose (Rodrigues

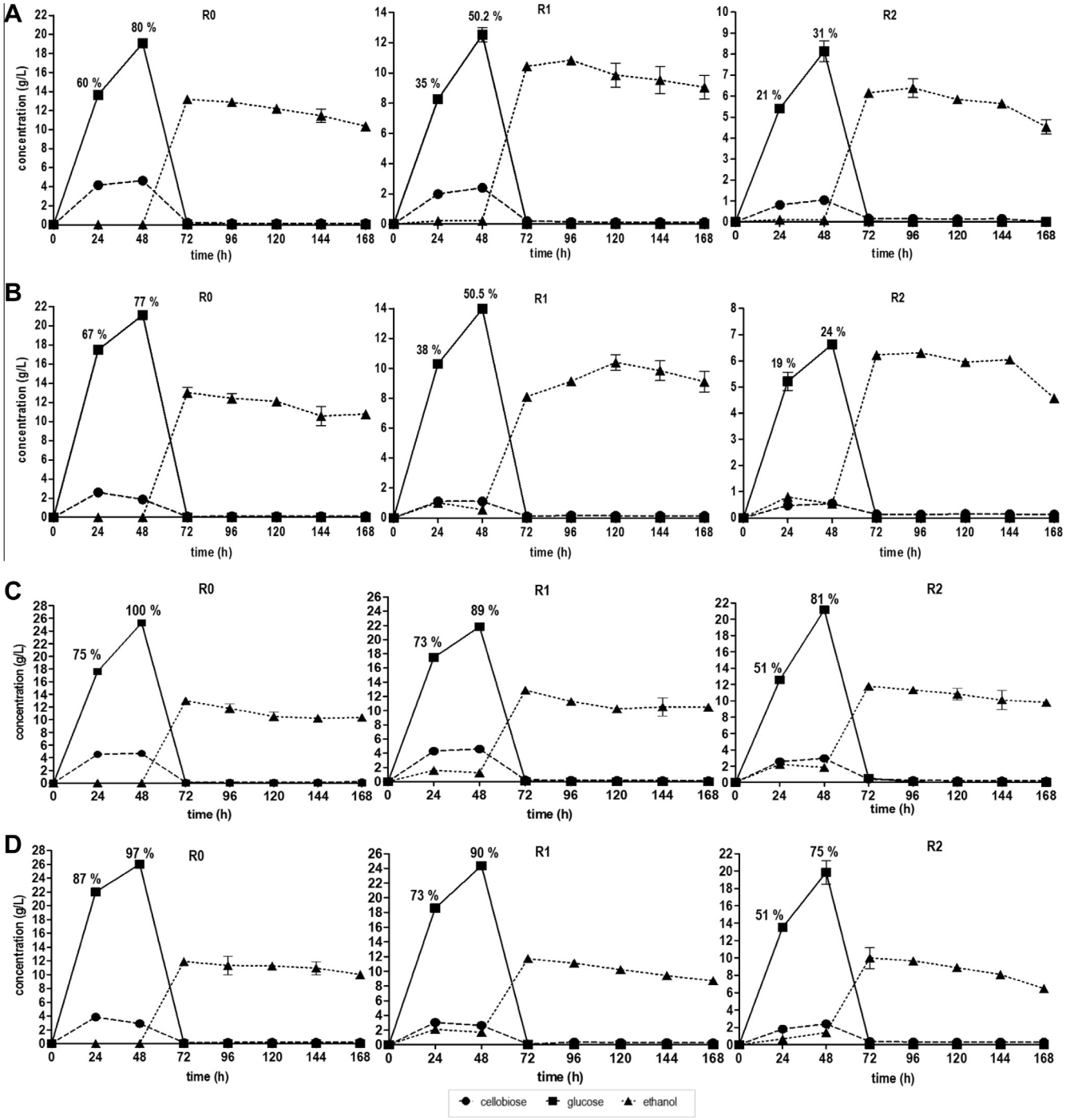

Fig. 2. Time course of the cellulose–sugars–ethanol conversion at, (A) 20 FPU/g cellulose, hydrolysis 37 °C, (B) 20 FPU/g cellulose, hydrolysis at 50 °C, (C) 40 FPU/g cellulose, hydrolysis at 37 °C, (D) 40 FPU/g cellulose, hydrolysis at 50 °C. R0, R1 and R2 refers respectively to the initial, first and second round of enzyme recycling, respectively. The cellulose conversion degrees at 24, 48 h, (%) are shown on the top of the graphs. Reported values are average of duplicates, error bars represent ± standard deviation.

et al., 2012). Therefore, it seems that reaching a high conversion degree is helpful in allowing the enzyme to become available in the liquid fraction, which obviously makes it easier to be recovered. A distinct behaviour is observed in the case of β-glucosidase, which is poorly adsorbed by the substrate (Fig. 1A–D), certainly due to the absence of a cellulose-binding domain in this enzyme.

3.2. Enzyme recovery

The results discussed in the previous section indicate that a substantial amount of enzyme activity can be recovered from the liquid fraction (superior to about 70%), provided a high conversion degree is achieved (as under the conditions corresponding to Fig. 1C). The soluble enzyme may be recovered by ultrafiltration. As shown in Table 1, about 20% (between 11% and 29% of the initial load) of the enzyme is lost in this operation, thus we have added 20% of fresh enzyme in the rounds R1 and R2. Although a membrane with a cut off weight of 10 kDa has been used, still some enzyme is lost due to filtration. Because, ultrafiltration process was conducted on relatively small samples in lab scale, the amount of material lost during handling, e.g. in the filtration device, becomes a large percentage. In an industrial scale loosing

Table 1
Fraction of Cel7A, Cel7B and β-glucosidase activities recovered (% of original load) and lost (% of original load) in each round after ultrafiltration step compared to the enzymatic activity recovered in the liquid fraction after fermentation.

Enzyme	Round	20 FPU/g cellulose, 37 °C prehydrolysis		20 FPU/g cellulose, 50 °C prehydrolysis		40 FPU/g cellulose, 37 °C prehydrolysis		40 FPU/g cellulose, 57 °C prehydrolysis	
		Enzyme recovery (% of original load)	Enzyme lost (% of original load)	Enzyme recovery (% of original load)	Enzyme lost (% of original load)	Enzyme recovery (% of original load)	Enzyme lost (% of original load)	Enzyme recovery (% of original load)	Enzyme lost (% of original load)
Cel7A	Initial(R0)	55	20	33	29	66	16	38	18
	1st round (R1)	46	21	28	33	67	14	32	39
	2nd round (R2)	38	29	39	11	60	18	39	32
Cel7B	Initial(R0)	54	24	31	28	60	20	42	14
	1st round (R1)	47	21	31	28	61	18	36	43
	2nd round (R2)	35	23	39	9	57	14	47	21
β-glucosidase	Initial(R0)	77	11	61	16	78	7	59	16
	1st round (R1)	77	14	67	6	73	14	69	8
	2nd round (R2)	71	18	75	7	71	19	67	17

enzymes due to material losses would naturally be a point of optimization. We believe that by using a lower MWCO and by operating at an industrial scale (where the loss of small amounts of sample has lower impact in the final recovery) better yields can be reached. It is interesting to note that the yield of activity recovery from the liquid fraction after ultrafiltration was much higher when lower temperatures of hydrolysis (37 °C) and high initial loading of enzyme (40 FPU) were used. Indeed, a final recovery of both Cel7A and Cel7B of about 60% (from 57% to 67% of the initial load) was obtained using those conditions (Table 1 R0, R1 and R2). Raising the hydrolysis temperature to 50 °C and 40 FPU resulted in a drop of the recovery from liquid fraction of the enzyme to about 40%, certainly due to the protein instability, which again resulted in lower substrate conversion (as discussed in the previous section). Consequently, less enzyme is recovered from liquid fraction due to inactivation and also because of higher adsorption to the solid residue. The effect of the hydrolysis temperature was confirmed by analysing the results obtained using 20 FPU.

Along with the recycling rounds, in particular for low enzyme loadings, higher amounts of enzyme remaining adsorbed on the solid fraction after fermentation are detectable. For example at 20 FPU and 37 °C, the adsorbed Cel7A after fermentation raises from 30% of the original load at R0 to 48% at R2; similar results are observed for Cel7B (Fig. 1A). This is probably because of the lower conversion degrees in the successive rounds of hydrolysis/fermentation. Indeed, as we have shown in a previous study (Rodrigues et al., 2012), Cel7A and Cel7B have higher affinity for cellulose than for lignin.

The enzyme activities shown in Table 1 were measured using low molecular weight substrates, specific for the different kinds of enzyme present in the mixture. In order to verify whether the recovered enzyme was fully functional – able to bind and hydrolyse insoluble cellulose fibres – a filter paper assay was carried out. Thus, it was observed that the enzyme recovered, retains its ability to bind and hydrolyse a fresh substrate. The results obtained generally confirm the previous discussion: a more significant reduction of activity is observed when a temperature of 50 °C is used during hydrolysis. In the case of the assay performed with 40 FPU and a hydrolysis temperature of 37 °C, the values of FPase activity unexpectedly increased in the consecutive rounds, probably because the concentration of β-glucosidase also increased and this may have had a significant impact on the results obtained using this assay (Table 2).

3.3. Time course of enzymatic hydrolysis

The activity profile of the various enzymes over time under the different conditions was monitored. It has to be recognized that quite high enzyme loadings were used (20 and 40 FPU). This was a choice in this work, made under the rational that it may pay to use high enzyme loads as long as the enzymes are recycled. Using more enzyme allows of course for a more efficient cellulose hydrolysis, as can be seen in Fig. 2A–D. As pointed out already, the effective conversion of cellulose allowed in turn for more enzyme to return to the liquid fraction, consequently allowing its easier recycling.

All of the experimental conditions showed a successive decrease, after the initial round R0, in the glucose yields detected at the end of the hydrolysis period (48 h) (Fig. 2A–D), an effect more severely observed for the assays carried out with 20 FPU. In spite of the enzyme deactivation observed at 50 °C, the use of 40 FPU affords for more effective conversion, as could be expected. Indeed, the residual Cel7A activity observed at 50 °C – 40 FPU (Fig. 1D R2), about 0.9 IU/mL, is slightly higher than the activity at the beginning of the process for 37 °C – 20 FPU, about 0.8 IU/mL (Fig. 1A R0).

The analysis of the insoluble residues obtained under the different conditions (Table 3) revealed that the estimation of cellulose conversion on the basis of soluble sugar analysis is probably overestimated, as still some glucose was detected in all of the insoluble residues obtained, although the soluble sugar analysis indicated yields of conversion close to 100%, in some cases, already at 48 h (Table 3). Only using 40 FPU and 37 °C, during the hydrolysis stage,

Table 2
Determination of FPase activities in the samples obtained after the consecutive rounds of hydrolysis (at time 0 and after 168 h, before ultrafiltration). In the case of samples labelled (*) it has not been possible to measure the FPase activity.

Conditions	20 FPU/g cellulose, 37 °C prehydrolysis		20 FPU/g cellulose, 50 °C prehydrolysis		40 FPU/g cellulose, 37 °C prehydrolysis		40 FPU/g cellulose, 50 °C prehydrolysis	
Round	0 h (FPU/mL)	168 h (FPU/mL)	0 h (FPU/mL)	168 h (FPU/mL)	0 h (FPU/mL)	168 h (FPU/mL)	0 h (FPU/mL)	168 h (FPU/mL)
Initial(R0)	0.89	0.74	0.81	0.55	1.51	1.27	1.39	1.09
1st round(R1)	0.86	0.53	0.65	0.44	1.64	1.44	0.91	0.86
2nd round(R2)	0.62	1.98*	0.47	1.69*	1.95	1.29	0.83	0.59

* mmoles glucose equivalents released per minute averaged over 60 min.

Table 3
Composition of lignocellulosic solid residue (% of dry weight) after hydrolysis and fermentation of wheat straw at different conditions.

Conditions	20 FPU/g cellulose, 37 °C prehydrolysis		20 FPU/g cellulose, 50 °C prehydrolysis		40 FPU/g cellulose, 37 °C prehydrolysis		40 FPU/g cellulose, 50 °C prehydrolysis	
Round	% Cellulose (glucan)	% Lignin Klason	% Cellulose (glucan)	% Lignin Klason	% Cellulose (glucan)	% Lignin Klason	% Cellulose (glucan)	% Lignin Klason
Initial(R0)	5.5	84.9	6.8	79.9	5.02	86.9	6.02	89.8
1st round(R1)	12.0	78.9	19.05	79.6	5.9	84.09	8.8	83.4
2nd round(R2)	26.05	65.9	31.1	58	9.4	80.2	12.3	77.4

similar yields of cellulose conversion (Table 3) and ethanol (Fig. 2) were obtained throughout the 3 rounds, although the residual cellulose was a bit higher for R2 than in the other rounds, and correspondingly also the ethanol concentration was a bit lower.

4. Conclusions

The conditions favoring a more efficient cellulose conversion also favor desorption of the enzymes, allowing easy enzyme recovery by recycling the liquid fraction. Ultrafiltration allows the recovery of the soluble enzyme with a yield of about 80%, in the lab scale trials. Operating at a temperature which does not compromise the enzyme stability is absolutely essential concerning recycling. Even when reaching high conversion degrees, still a relevant amount of enzyme remains attached to the final residue (at least 20–30%). Finally, as an overall conclusion: enzyme recycling is certainly a possibility requiring demonstration in larger scale trials.

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