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Influence of enzyme loading on enzymatic hydrolysis of cardboard waste and size distribution of the resulting fiber residue

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Abstract

Enzymatic hydrolysis of lignocellulosic biomass to sugars alters the properties of the cellulosic fibers. Several process variables, including enzyme loading, play an important role in these changes. Many physical properties of fibers are affected: their length and width, porosity, specific surface area, and degree of fibrillation, for instance, may undergo dramatic changes when subjected to enzymatic degradation. In this study, the influence of enzyme loading on the fiber size was investigated using milled cardboard waste as the raw material. The effect of cellulases and hemicellulases on the monosaccharide production and the resulting fiber size was studied using commercial enzyme products. It was shown that the cellulase loading largely determined the amount of sugars produced. The fiber length was reduced during the course of hydrolysis, although the size reduction was not especially dramatic. Based on the SEM images, no significant damage to the fiber surfaces occurred during the process.

Keywords *Hydrolysis, Enzyme loading, Cellulase, Fiber size, Cardboard*

1. Introduction

Various lignocellulosic wastes, residues and crops are under consideration for industrial bioethanol production, in part due to their relatively high cellulose contents and excellent availability. Driven by rising oil prices and the increasing demand for sustainably-produced transportation fuels, the first commercial bioethanol plants for the demonstration of industrial production have recently been constructed (Larsen et al., 2012; Huang et al., 2009). Enzymatic hydrolysis, performed in order to cleave cellulosic polymers to monosaccharides, has been recognized as the key process stage to enable feasible bioethanol production. On the other hand, the main difficulties in the process are related to this challenging process stage which, together with required pretreatment and enzyme production, may contribute to over 40 % of the total cost of bioethanol production (Banerjee et al., 2010). Several process configurations for bioethanol production have been proposed, but a number of technical difficulties remain (Cardona and Sanchez, 2007; Hamelinck et al., 2005; Huang et al., 2008).

Cellulases and hemicellulases are the enzymes most typically used to cleave cellulose and hemicellulose to monosaccharides. There are many characteristics that affect the enzyme choice and requirement, such as the substrate type, composition and lignin content (Van

Dyk and Pletsche, 2012). The practicality of enzyme loading, in turn, is determined by cost factors, i.e. the cost of enzyme and the price of the end product, and can be optimized (Newman et al., 2013). In the case of industrial processes, in which a high sugar concentration must be obtained, the initial suspended solid concentration in the hydrolysis should be high, preferably over 200 g solids / kg suspension. However, high solid loadings are known to reduce the obtainable yield (Kristensen et al., 2009), which adversely affects the process economy.

Development of effective bioethanol production from lignocellulosic raw materials can be facilitated by an in-depth understanding of fiber properties during the enzymatic saccharification. During the degradation of solid biomass (cellulose and hemicelluloses) to their structural sugars, the suspended solids content in the solid-liquid system decreases while the concentration of dissolved solids correspondingly increases. The changes in the physical and chemical composition of the biomass, as well as rheological characteristics of the suspension (Nguyen et al., 2013), may be dramatic.

Previous studies have shown that the both the initial composition and upstream pretreatment of the feedstock have a large influence on the success of enzymatic hydrolysis. The selection of the pretreatment method is influenced, for instance, by the type of raw material and the costs of enzymes (Jorgensen et al., 2007). Pretreatment with steam and/or acids has been widely applied and recognized as effective, in spite of some drawbacks, such as formation of inhibitory compounds (Galbe and Zacchi, 2012). Reduction of the particle size and fiber dimensions of the biomass may improve enzymatic saccharification greatly in many cases (Hoeger et al., 2013; Yeh et al., 2010) but not without exceptions (Del Rio et al., 2012). The enzyme loading also has an important role in the process (Soares et al., 2011). Although high enzyme loading typically results in an enhanced degree of conversion, the relative improvement may be rather poor (Kinnarinen et al., 2012). The main focus of the intensive research on enzymatic hydrolysis has been on the pretreatment stage. However, there are some interesting industrial waste fractions, such as cardboard waste, that can be hydrolyzed even without any other pretreatment than particle size reduction. Consequently, when there is no pretreatment stage, the hemicellulosic sugars can be potentially recovered simultaneously with the main hydrolysis product, glucose. In this process, not only the saccharification itself is interesting, but the the properties of non-degraded fiber residue largely determine its potential for utilization. The fiber residue can be pumped into the fermentation stage, recycled back to hydrolysis, perhaps after enzyme recovery by desorption (Moniruzzaman et al., 1997), deliquored (Kinnarinen et al., 2012) and dried for combustion, or utilized in some other way. Selection of the utilization method depends on the fiber properties, from which the size of the fibers is particularly important. Changes in fiber size during the enzymatic hydrolysis of lignocellulosic biomass have, up to date, only been evaluated in a few studies. Most of the previous attempts to study the subject have been made using strongly diluted fiber suspensions, which may have led to excessively high sugar yields and overestimation of the fiber length reduction, as compared to hydrolysis at more realistic solid loadings. The mechanism of fiber length reduction, caused by enzymatic attack, has been recently investigated by Clarke et al. (2011), who observed substantial fiber cutting already during the initial period of hydrolysis.

In this study, the influences of cellulase and hemicellulase loading on the degree of saccharification and fiber size were investigated. Commercially available cellulase and hemicellulase preparations (Cellic CTec2 and HTec, Novozymes, Denmark) were used. The fiber size distributions during the 24 hours of hydrolysis were determined using a standard fiber testing instrument, in which the fibers were measured with an image analysis technique. In order to determine the respective sugar concentrations, the liquid phases of the same samples were analyzed using high-performance liquid chromatography (HPLC). Additionally, visual characterization of the fibers was performed using scanning electron microscopy (SEM).

2. Materials and methods

2.1. Composition of cardboard waste

In the experiments, air-dry cellulosic waste was used as the raw material. The raw material consisted mainly of shredded corrugated cardboard, collected from Finland. In addition to cellulose, hemicelluloses and lignin, several impurities were present: pieces of plastic, metals and inorganic minerals were observed in the raw material. Prior to the analyses and experiments, the sample was milled, using a hammer mill, in order to reduce its particle size. The initial particle size is presented in the Results and Discussion Section. An approximate chemical composition of the raw material is presented in Table 1.

Table 1. Approximate composition of the cardboard waste used as the raw material for the experiments.

Component	Concentration wt.%	Method or reference
Cellulose	63 ± 1.6	Black [26]
Hemicellulose	14	Calculated
Lignin	12 ± 0.4	Kinnarinen et al. [22]
Ash	11 ± 0.2	ISO 1762:2001

The composition was comparable to that of old corrugated cardboard, reported by Yañez et al. (2004). The cellulose content of the raw material was determined according to the method of Black (1951), utilizing the anthrone reagent in strong sulfuric acid. The proportion of lignin was measured using a liquid chromatographic method (Phenomenex Luna 3u C18(2) column, 20 mM ammonium hydroxide / methanol, 50/50 vol-%, as eluent), described in more detail by Kinnarinen et al. (2012). The hemicellulose content was calculated, not measured, assuming that no extractives were present. Due to the presence of inorganic matter, such as calcium carbonate, used as a filler in paper and cardboard products, the ash content was relatively high. The ash content was determined according to the ISO 1762:2001 standard.

2.2. Preparation of fiber suspensions

The fiber suspensions of 10 wt.% were prepared in sealed plastic bottles ($V = 50 \text{ cm}^3$). In each bottle, the same weight of the milled raw material was added and extremely pure RO water (Millipore) was added to form the suspension. After thorough mixing, in a shaker, for one hour, sulfuric acid (2.0 mol dm^{-3}) was added to the bottles to gradually adjust the pH to 5.0. In order to obtain an optimum temperature for the hydrolysis experiments, a water bath with bottle holders was prepared and set to a constant temperature of $46.0 \text{ }^\circ\text{C}$, which was at an optimum level for the process, according to the enzyme manufacturer.

The enzymes used were commercial preparations (Novozymes, Denmark), consisting of different types of enzymes designed to degrade cellulose and hemicellulose. The cellulase was of the CTec2 type, while the product type of the hemicellulase was HTec. The activity of the cellulase product was not measured, but it was approximately 150 FPU cm^{-3} , according to Zhou et al. (2013). Based on literature (Eckard et al., 2012), the xylanase activity of Cellic HTec was 1090 FXU cm^{-3} . Both cellulase and hemicellulase were added simultaneously into the bottles, after which the bottles were closed, shaken manually for 30 s and placed in the temperature-controlled water bath. The experimental plan with the applied enzyme loadings is shown in Table 2.

Table 2. Experimental plan. The enzyme concentrations are given in relation to the mass of dry raw material.

Test	Enzyme concentration	
	CTec2* $\text{cm}^3 \text{ kg}^{-1}$	HTec $\text{cm}^3 \text{ kg}^{-1}$
1	192	5
2	192	10
3	192	20
4	192	40
5	24	40
6	48	40
7	96	40
8	192	40

*Estimated activity approximately 150 FPU cm^{-3} (Zhou et al., 2013)

The enzyme loadings were selected based on prior experience to obtain effective saccharification and enable easy sampling already during the initial period of hydrolysis.

2.3. Experimental procedure

In typical saccharification and fermentation processes for lignocellulosic materials, either separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF) is applied for 48 – 72 h. This study aimed at investigating specifically the initial 24 h period of enzymatic hydrolysis, during which time, based on prior experiments, most changes in the fibers typically take place. The procedure for sample preparation and analytical operations is illustrated in Fig. 1.

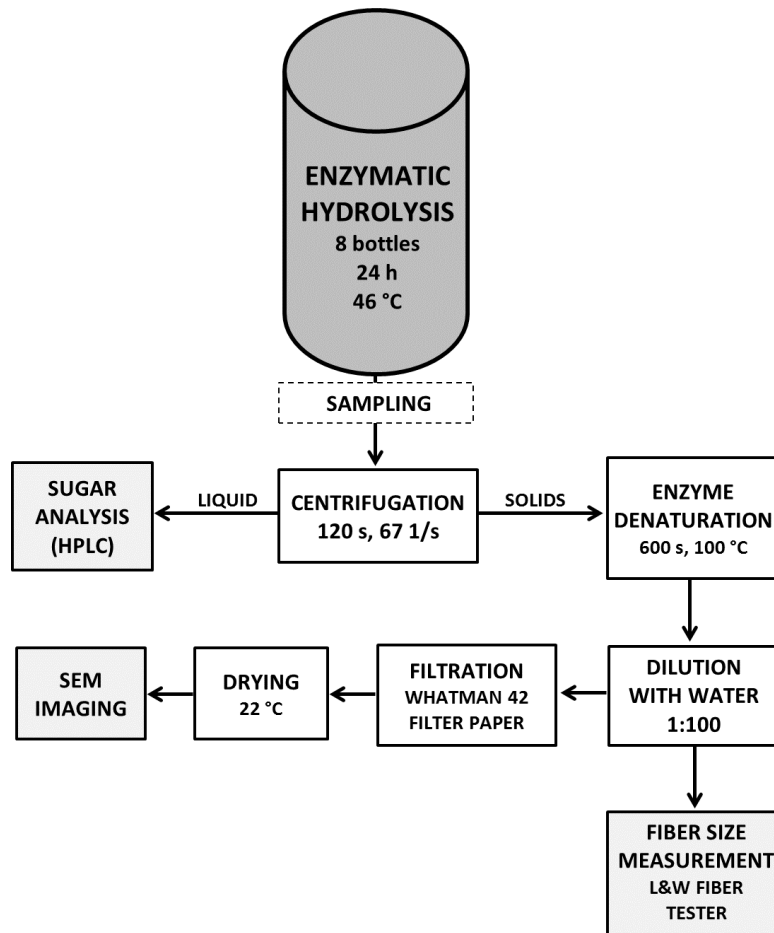


Fig. 1. The experimental procedure.

Six samples were taken from each suspension: one before the enzyme addition and five during the hydrolysis period of 24 hours. Prior to sampling, the bottles were shaken to homogenize the suspension. The sampling was performed using conventional Pasteur pipettes (3 cm³), cut accordingly in order to obtain a significantly enlarged inlet/outlet diameter, which also enabled sampling from the thick, less hydrolyzed suspensions. The total volume of each sample, transferred into plastic test tubes for centrifugation, was intended to be 3 – 5 cm³. A Jouan GT 422 bucket centrifuge, equipped with the appropriate

holders for the test tubes, was used for separating the suspension into liquid supernatant and a settled fiber bed. The centrifugation was continued at 67 Hz for 120 s.

The supernatant was filtered through a syringe filter with a nominal pore size of 0.2 μm and analyzed for monosaccharides with high-performance liquid chromatography (HPLC). The HPLC procedure is described in Section 2.5.

In order to avoid further degradation of fibers in the test tubes, the enzymes were denatured at 100 °C. The denaturation was performed by keeping the test tubes in a bath of boiling water for 600 s. The fiber samples were then dispersed in water, first in the test tubes with 5 cm^3 of Millipore water prior to placing the samples in a freezer. Prior to fiber size measurement, the samples were again diluted in water in order to separate the fibers from each other. After the fiber size measurements, these dilute suspensions were filtered through Whatman 42 filter paper and dried at room temperature to obtain a solid and relatively flat bed of fibers for scanning electron microscopy (SEM) imaging.

2.4. *Fiber characterization*

Size distributions of the fiber suspensions at different times during the hydrolysis were determined using a Lorentzen & Wettre fiber tester (Kista, Sweden). The main advantage of this instrument is that both the length and width distribution of the fibers is measured using a two-dimensional image analysis technique. The unit incorporates a very narrow gap between two plates in which the fibers are aligned so that the real dimensions of whole fibers can be measured from the images taken. As a result of milling the raw material prior to the experiments, the fiber length was reduced. This is the main reason why there are large amounts of fibers shorter than those obtainable from fresh wood. The maximum possible measurement range, according to the manufacturer, for the fiber length was 0.2 to 7.5 mm ($\pm 0.5\%$) and the range for the fiber width was 10 to 100 μm ($\pm 0.5\%$). All fibers shorter than 0.2 mm were classified as fines. In order to avoid emphasizing the small fibers, due to their high population, weighted distributions for the fiber length and width were calculated as described in previously by other authors, such as Robertson et al. (1999).

The mean length (L_m) and width of the fibers were determined from length-weighted distributions, which are commonly used in fiber analysis, because the relative number of fine fibers is too high for creating representative number-based population distributions. The calculations of fiber mean size and size distributions were therefore performed according to the principle presented in Eq. (1) and (2), showing the calculations related to the fiber length:

$$L_m = \frac{\sum_{i=1}^N n_i l_i^2}{\sum_{i=1}^N n_i l_i} \quad (1)$$

$$P_{l,i}(\%) = \frac{n_i l_i}{\sum_{j=1}^N n_j l_j} \quad (2)$$

, where n_i is the number of measured fibers in each length fraction and l_i is the average length of fibers in a fraction. $P_{l,i}$ is the percentage of length in category i in which the number of fibers is n_i and the average length of fibers is l_i .

A JEOL JSM-5800 scanning electron microscope with a voltage of 15 kV and a magnification of 200x was used for the evaluation of the appearance of the fibers before, during and after the hydrolysis.

2.5. Determination of sugar concentrations

Samples taken from the supernatant after the centrifugation of the primary sample (Fig. 1) were analyzed for monosaccharides. The concentrations of glucose and xylose in the produced hydrolysates were determined by high performance liquid chromatography (HPLC, HP Agilent 1100). A dilute ($0.005 \text{ mol dm}^{-3}$) aqueous solution of sulfuric acid was used as the eluent while the column (Varian Metacarb 87H) was kept at $60 \text{ }^\circ\text{C}$. The eluent flow rate was $0.01 \text{ cm}^3 \text{ s}^{-1}$. The injection volume was 10^{-6} dm^3 and a precolumn was connected to the feed line to protect the column from solid particles.

3. Results and discussion

The effect of cellulase and hemicellulase loading on the degradation of cardboard waste to glucose and xylose, was studied using a wide range of enzyme loadings. In Fig. 2a, the hemicellulase loading (volume of enzyme / weight of biomass) was constant, $40 \text{ cm}^3 \text{ kg}^{-1}$, and the effect of cellulase loading on the glucose production was investigated. Based on Fig. 2a, it is clear that the rate of enzymatic hydrolysis slows rapidly after the first hours of hydrolysis. Another apparent conclusion from Fig. 2a is that the enzyme consumption increases relatively more sharply than the obtained glucose concentration: doubling the enzyme loading does not result in a doubling of the glucose concentration of the hydrolysate. The final yields after 24 hours of hydrolysis ranged from 0.36 to $0.63 \text{ kg}_{\text{glucose}} \text{ kg}^{-1}_{\text{cellulose}}$. As can be seen in the experimental plan, Tests 4 and 8 were identical. This test was performed twice in order to evaluate the repeatability of the experiments. The final yields obtained in Tests 4 and 8 were 626 and $629 \text{ g}_{\text{glucose}} \text{ kg}^{-1}_{\text{cellulose}}$, respectively, which indicates good repeatability.

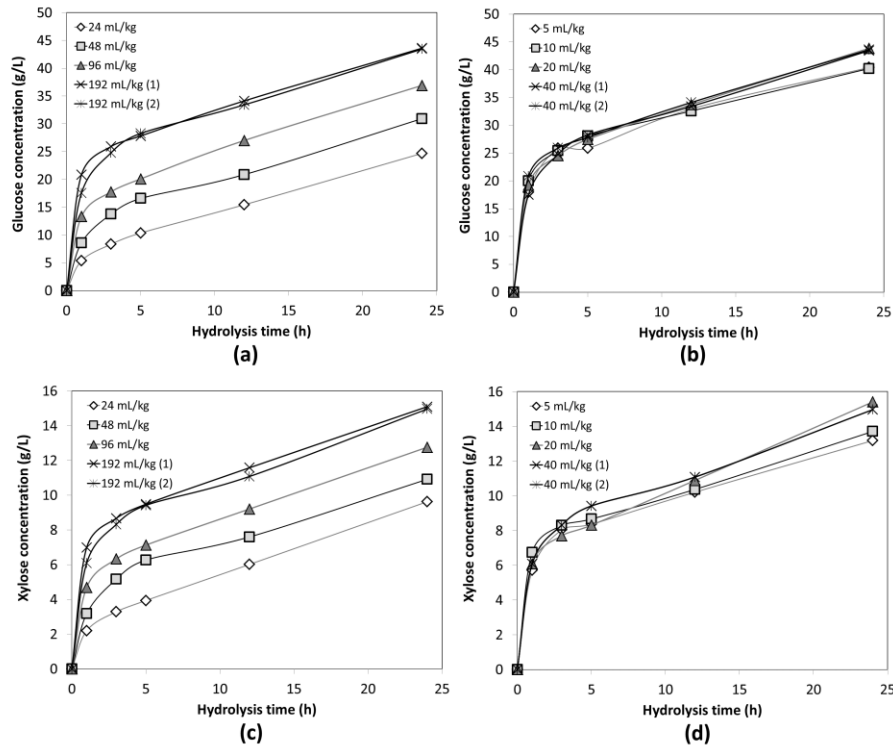


Fig. 2. Effect of cellulase (a) and hemicellulase (b) loading on conversion of cellulose to glucose. The hemicellulase loading in Fig. 2a was $40 \text{ cm}^3 \text{ kg}^{-1}$ and the cellulase loading in Fig. 2b was $192 \text{ cm}^3 \text{ kg}^{-1}$. Effect of cellulase (c) and hemicellulase (d) loading ($\text{cm}^3 \text{ kg}^{-1}$) on conversion of xylan to xylose. The hemicellulase loading in Fig. 2c was $40 \text{ cm}^3 \text{ kg}^{-1}$ and the cellulase loading in Fig. 2d was $192 \text{ cm}^3 \text{ kg}^{-1}$.

The influence of hemicellulase loading on glucose production is shown in Fig. 2b. It is probable that in this case hemicellulase is not a major obstacle for the conversion of cellulose to glucose. The high cellulase loading may be the most important reason for the weak improvement in the hydrolysis of cellulose that was obtained as a result of increased hemicellulase loading.

In Fig. 2c-d, the impacts of cellulase and hemicellulase loading on the xylose concentration of the hydrolysate are summarized. It is clear from Fig. 2c that degradation of cellulose was mainly responsible for increasing the availability of hemicelluloses for enzymatic degradation. Therefore, the hemicellulase loading (Fig. 2d) was of minor importance for hemicellulose conversion. These results imply that the cellulase preparation CTec2 itself, taking into consideration its high dosage, was so effective in hydrolyzing both cellulose and hemicellulose that the effect of hemicellulase addition becomes negligible. It is difficult to find the measured xylanase activity of Cellic CTec2 in the existing literature. However, results of previous studies (Zhou et al., 2013; Chen et al., 2013) indicate that this enzyme product has significant xylanase activity.

Hu et al. (2011) obtained clear improvements in the glucose and xylose yield using a mixture of commercial cellulase (Celluclast 1.5L) and β -glucosidase (Novozym 188) with

xylanase (Multifect Xylanase) addition in the hydrolysis of steam-pretreated corn stover. The xylanase activity of the Multifect xylanase was 2600 U cm^{-3} and the Celluclast product had also significant (440 U cm^{-3}) xylanase activity. The results of Hu et al. (2011) indicated that the positive effect of xylanase addition was more significant when the cellulase dosage was low. This observation supports the results of the present study, where the applied cellulase dosages were exceptionally high and hemicellulose addition did not improve the hydrolysis significantly. The results of Lin et al. (2010), obtained using artificial biomass (prepared from MCC from wood pulp, and xylan and lignin from birch wood) and commercial cellulase (ACCELLERASE 1500) and xylanase (OPTIMASH™ BG) showed a relatively small improvement in the xylose yield. According to Lin et al. (2010), the former of these enzyme products contained also hemicellulases. Generally, the optimum cellulase/hemicellulase ratio seems to depend on the properties of the substrate, the amount of enzyme(s) used and, regarding commercial enzyme preparations, the composition of the cellulase or hemicellulase product.

There are three major phenomena that have been proposed to restrict the sugar release and to cause the typical shape of the sugar concentration curves, seen in Fig. 2: limited accessibility of cellulose, which is also related to the lignin content, deactivation of enzymes during hydrolysis, and product inhibition caused by sugars (MacLellan, 2010). In certain situations, for pretreated suspensions in particular, the negative effect of lignin on the hydrolysis has been shown to be smaller than the influence of cellulose accessibility (Wiman et al., 2012). In the case of the cardboard waste used in the present study, the cellulose content of the substrate is high and the lignin content is lower than that of fresh biomass substrates. The matrix of carbohydrate polymers and lignin has also been opened in the pulping process. However, enzymatic hydrolysis of cardboard waste to sugars is hampered by the fiber hornification, i.e. reduction of the water binding capacity of the fibers, resulting from fiber drying. This reduces the enzyme accessibility, which is one important reason for the relatively low yield obtained at reasonable enzyme loadings.

In most studies related to enzyme loading, or enzymatic hydrolysis in general, high amounts of enzymes have been used. The enzyme dosages used in the present study were also too high for a commercial process. In order to enable commercially viable saccharification, significantly lower enzyme loadings should be used. This can be achieved by proper pretreatment and by recycling the enzymes. Adsorption and desorption of enzymes for the purpose of enzyme recycling have been studied in some previous articles (Kristensen et al., 2007; Weiss et al., 2013).

The effect of cellulase loading on the mean length and width of fibers during enzymatic hydrolysis is illustrated in Fig. 3a-b.

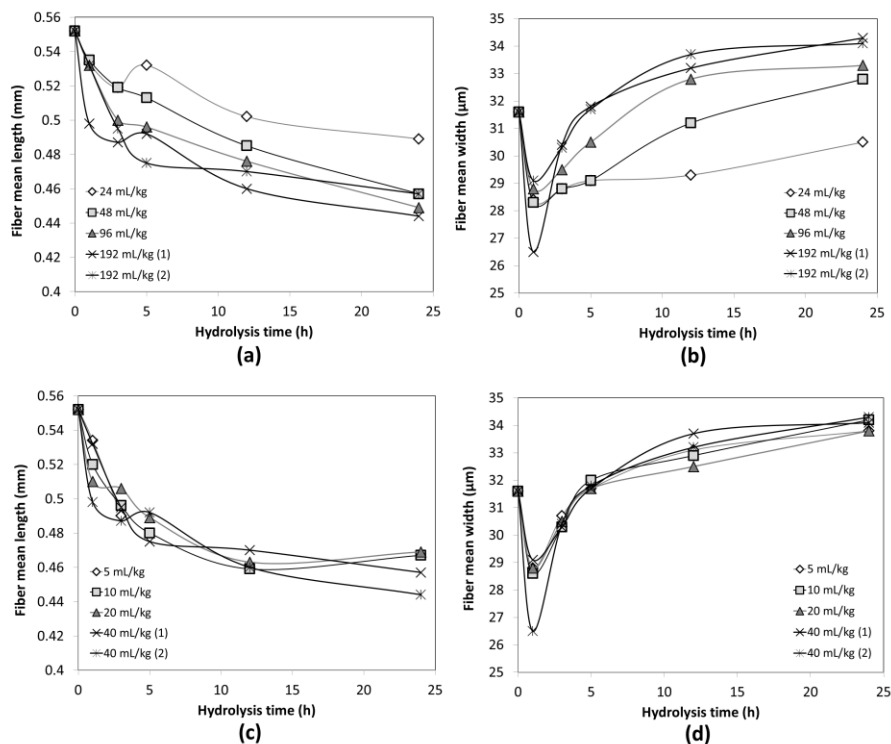


Fig. 3. Effect of cellulase loading ($\text{cm}^3 \text{kg}^{-1}$) on the fiber mean length (a) and width (b) during the 24 h hydrolysis period when the hemicellulase loading was $40 \text{ cm}^3 \text{kg}^{-1}$. Effect of hemicellulase loading ($\text{cm}^3 \text{kg}^{-1}$) on the fiber mean length (c) and width (d) during the 24 h hydrolysis period. The cellulase loading was $192 \text{ cm}^3 \text{kg}^{-1}$.

It can be observed in Fig. 3a that there is, at most, a reduction of approximately 20 % in the fiber mean length. The lowest enzyme loading of $24 \text{ cm}^3 \text{kg}^{-1}$ seems to result in smaller changes in the fiber length, which can be clearly observed after the initial 5 hours of hydrolysis. On the other hand, it is difficult to say whether the slight increase in the measured mean length in the following two cases is only random variation resulting from sampling and measurement errors.

In Fig. 3b, the shape of the graphs presenting the fiber mean widths during the hydrolysis is very interesting. As could be expected, the mean width of the fibers started to decrease rapidly after the enzyme addition. What happened after this very initial period is likely to result from swelling of fibers, increased fibrillation, which could increase the measurable fiber width, or simply size reduction of small fibers to such an extent that they were classified as fines, and therefore excluded from the width distribution.

Under those experimental conditions where the cellulase loading was exceptionally high, hemicellulase addition was not significantly responsible for a reduction in the fiber length (Fig. 3c). The use of high cellulase loading caused an initial reduction in the fiber width, after which the fiber width increased slowly by about 17 % (Fig. 3d). The hemicellulase loading was not observed to be of any importance in this process.

Unlike most previous studies, the present study was performed in non-mixed conditions, in order to investigate the sole effect of enzymatic hydrolysis on the fiber dimensions. Factors other than the enzyme loading also appear to have an influence on the fiber size. Samaniuk et al. (2011) studied the effect of mixing on the average fiber length during the very initial stage of enzymatic saccharification. Their mixing experiments were performed using a modified torque rheometer equipped with motorized counter-rotating screw elements. They also made control experiments, without mixing, and observed that the average fiber length was not significantly affected by the enzymes when the reaction suspension was not mixed. Because the duration of hydrolysis in the study of Samaniuk et al. (2011) was only 40 minutes, while it was 24 hours in the present study, the results are not well comparable. Chinga-Carrasco et al. (2010) studied the same topic from a microscopic point of view by monitoring submicron-scale changes in Kraft pulp fiber surface during enzymatic hydrolysis carried out in shaken flasks. It was noticed in their study that enzymatic hydrolysis using Novozym 188 resulted in changes in the fibrillar structure of the fibers, including changes in the morphology and reduction of anisotropy, i.e. a measure of degree of orientation of the surface texture of the fibers. These changes may affect the behavior (e.g. the rheology and the water retention capacity) of the fiber suspension in the subsequent process stages more than the macroscopic size reduction of the fibers. In an earlier study (Ramos et al., 1993), a rapid size reduction of eucalyptus fibers was obtained, which consequently increased the proportion of fines in the suspension. The rapid size reduction obtained in the beginning of hydrolysis is in line with the results of the present study.

In papermaking, enzymatic modification of fibers has been performed for decades. The aim in this case is to make the pulp properties more beneficial, while avoiding conversion of high amounts of cellulose to sugars. The cutting of fibers to shorter fragments cannot typically be completely avoided when cellulases are used. Pere et al. (1995) used two different cellobiohydrolases and endoglucanases produced by *T. reesei* for the modification of unbleached pine kraft pulp. They obtained neither a significant yield of hydrolysis nor reduction in the fiber length, but the viscosity was significantly reduced. The primary reason for the small change in the fiber length may have been the low enzyme dosage. However, dramatic changes in the pulp viscosity were observed, which was mainly due to the presence of endoglucanases. With respect to the viscosity, similar observations have been reported in previous articles (Samaniuk et al., 2011; Nguyen et al., 2013).

As regards the fiber size distributions, clear changes in the shape of the differential length-weighted distribution occurred during the hydrolysis. The reduction in the fiber length is readily observed (Fig. 4a). Most of the length reduction took place during the first three hours of hydrolysis and the shape of the distribution became narrower and more regular as the largest fibers were hydrolyzed. Consequently, the peak height of the length distribution was increased by 60 % during the hydrolysis time of 24 h. The final fiber length distributions after 24 h, on the other hand, were not much influenced by the enzyme loading, apart from one exception (Fig. 4b).

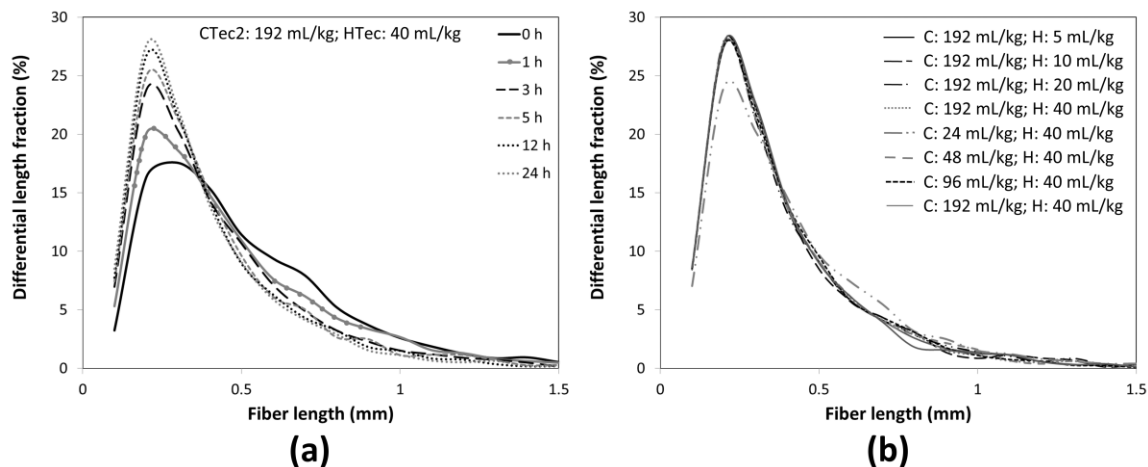


Fig. 4. Test 4: Changes in the fiber length distribution during the 24 h hydrolysis period at the highest enzyme loading, CTec2 192 $\text{cm}^3 \text{kg}^{-1}$ / HTec 40 $\text{cm}^3 \text{kg}^{-1}$ (a). All experiments: influence of enzyme loading ($\text{cm}^3 \text{kg}^{-1}$) on the final fiber size distribution after 24 h of hydrolysis (b).

It is apparent that the lowest cellulase loading used in Test 5 was insufficient to affect the fiber length as effectively as the higher loadings. It can thus be concluded that the general level of cellulase loadings in this study was too high to produce observable differences, i.e. the length reduction was at the maximum level obtainable using the enzyme products in question.

A theory on fiber cutting during enzymatic hydrolysis of lignin-free fibers has been proposed by Clarke et al. (2011). They recognized certain dislocation sites, where the fiber cutting took place, while the decrease in the fiber length was extremely significant. The results of Clarke et al. (2011) show the essential importance of the first hours of hydrolysis for fiber size reduction, which supports the findings of the present study. However, the magnitude of size reduction in their study was greater, which is likely to have resulted from the absence of lignin and the low substrate loading (20 g kg^{-1}).

Based on the results presented in Figs. 2-3, the repeatability of the experiments seems to be good. The largest uncertainty in the results is related to the early stage of hydrolysis, when the sampling error was probably larger than in the end of hydrolysis.

The fiber size analyzer was used to measure the fiber lengths within a range of 0.2 – 7.5 mm. All fibers shorter than 0.2 mm were classified as fines, the proportion of which increased during hydrolysis. The proportion of fines rose from the original 24 % to 58 % in the case of the highest cellulase loading and to 45 % in the case of the lowest cellulase loading.

SEM images of the original fibers, prior to and during hydrolysis, are presented as electronic supplementary material.

When it comes to the surface of the fibers, no clear changes can be observed. This can result from the drying procedure used for the sample preparation, which causes shrinkage of fibers and may, therefore, hide the most apparent changes. However, there may be some

changes in the structure of the fiber network. It appears that the most hydrolyzed fibers form a denser and flatter network, compared to the other cases. This could result from the decrease of the fiber strength, which in turn is mostly caused by hydrolysis of the main supporting component, cellulose. It is difficult to make more detailed conclusions from the SEM images, because in this case the images could only be visually evaluated. In order to analyze the SEM images, possibly to associate SEM imaging with the fiber size data, advanced software should be used.

Conclusions

In this study, the effects of enzyme loading on the saccharification of cardboard waste and characteristics of the residual fibers have been investigated. The results show that the reduction of fiber size is moderate and takes place very rapidly after the addition of enzymes. Visual observations from the SEM images supported these conclusions. Cellulase was observed to be responsible for most changes in the extent of saccharification and fiber length reduction. Future studies could focus on more detailed characterization of partially hydrolyzed fibers. The influence of impurities (minerals, ink, lignin, etc.) on the enzyme requirement could also be evaluated.

Supplementary data

SEM images of the fibers before and during the hydrolysis are available as online supplementary material.

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Supplementary material

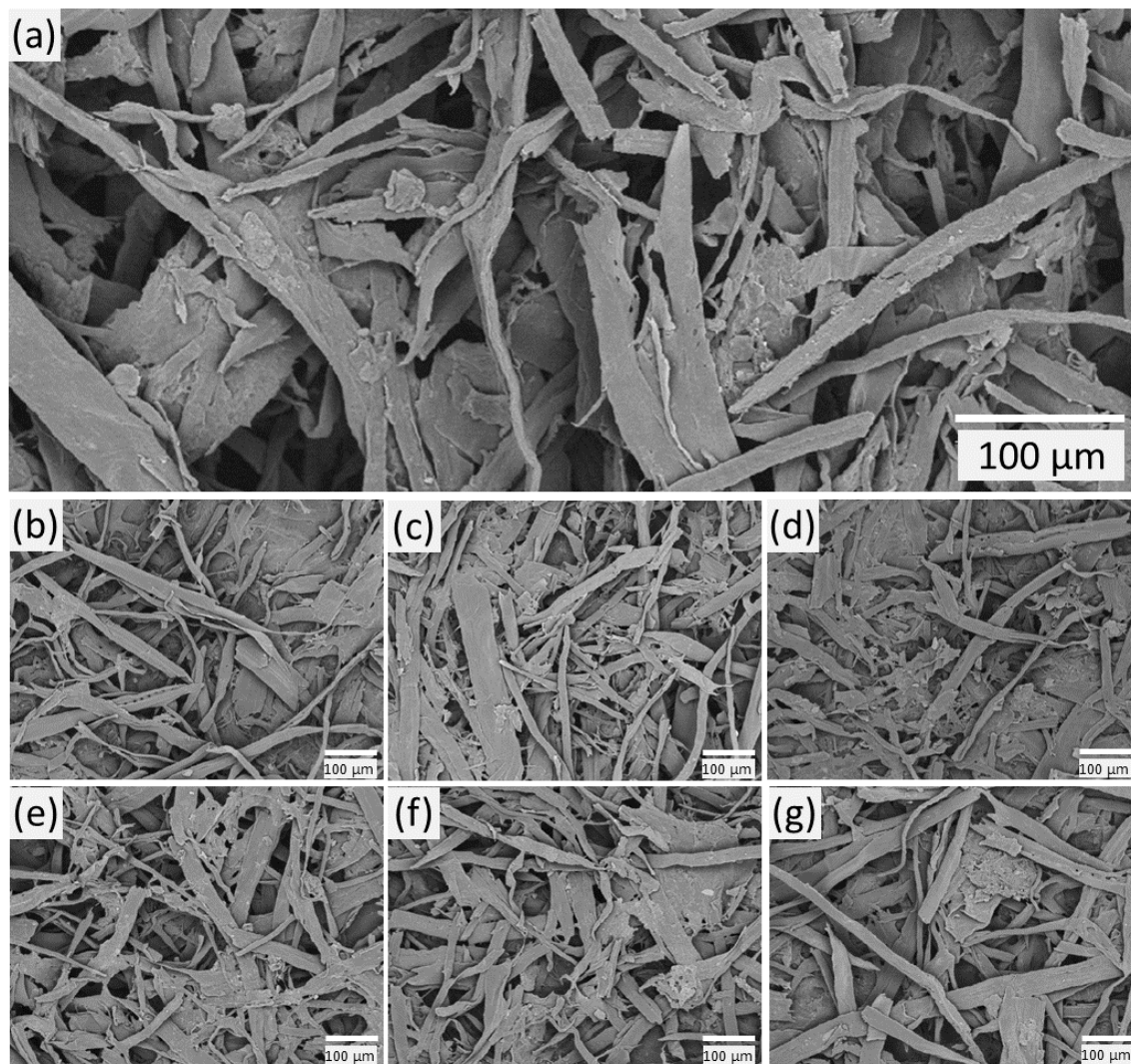


Fig. S1. SEM images (200 x magnification) of the original fibers (a) and fibers after 1 h (b,e), 5 h (c,f), and 24 h (d,g) of enzymatic hydrolysis at the highest (Test 4: b-d) and lowest (Test 5: e-g) enzyme loadings.