The role of the particle size reduction and morphological changes of solid substrate in the ultrasound-aided enzymatic hydrolysis of cellulose

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A B S T R A C T

The contribution of ultrasound-aided particle size reduction to the efficiency of the subsequent enzymatic hydrolysis and the accompanying morphological changes of bleached cotton and linen powders were investigated. The aqueous suspensions of cellulosic powders were pretreated either with an ultrasonic bath (US-B) or with a horn-type reactor (US-H). Results revealed that the impact of US-H was more pronounced than that of the US-B. Clearly, the linen particles were more sensitive to ultrasonication than cotton. The US-H modified the particle size distribution differently for the cotton and linen powders and reduced the mean size of particles from 49 to 40 µm and from 123 to 63 µm, respectively. A significant increase in the water retention and water sorption capacity was also measured. The smaller particles with increased accessibility were preferably digested in the enzyme treatment, resulting in a considerably higher concentration of reducing sugars and an enrichment of the residual particles with a larger average size (cotton: 47 µm; linen: 66 µm).

1. Introduction

Owing to the growing environmental awareness, the extraction of cellulose from the lignocellulose-based biomass has great industrial importance. The conversion of cellulosic into valuable products and their utilization have attracted significant attention and generated innovations in several industrial sectors. Conversion by enzymes has huge potential since the enzymes are highly selective and act at low temperatures and near-neutral pH. This biotechnological process is essential in the fuel, food and chemical industries [1,2].

The rate and extent of the heterogeneous enzymatic hydrolysis of cellulose were widely investigated. Despite the synergy of enzymes (i.e. endo- and exo-glucanases, β-glucosidase) the hydrolysis of cellulose is a time-consuming process. The action of enzymes on cellulose may be affected by various factors including the properties of the solid substrate (format and size, morphology, type and amount of accompanying non-cellulosic components, etc.), the application and intensity of different agitating systems (magnetic stirring, horizontal shaking, vertical agitation, etc.) and the parameters of hydrolysis (substrate and enzyme loading, temperature, duration, etc.) [3–5]. Among the several factors that can have a significant effect on the rate of enzyme-catalyzed depolymerization, the substrate properties were found to be limiting, since the accessibility of cellulose to the enzyme depends on the physical–chemical and structural parameters of the cellulosic material [6,7].

The accessible specific surface area of cellulose is determined by the particle size and pore volume of substrates. The reduction of particles size by various grinding and milling methods is often associated with the disruption of the original ordered structure of cellulosic materials, resulting in a decrease in crystallinity and degree of polymerization, and an increase in water sorption capacity. These changes are beneficial for the enzyme-catalysed digestibility of cellulose [8,9].

Several publications report that the synergic action of endo- and exo-cellulases on the solid surface of cellulosic materials can be accelerated by ultrasonication with frequencies of 20–100 kHz. This low-frequency or power ultrasound is associated with acoustic cavitation. Questions addressing the potential benefits of ultrasonication in the heterogeneous enzymatic hydrolysis of pure cellulose and cellulose in biomass have become more complex since additional factors, also significantly affecting the process, are involved including the amplitude of ultrasound, duty cycle, presence/absence of a rigid reflector in the sonicated vessel. Nevertheless, the positive contribution of power ultrasound to the enzyme catalyzed heterogeneous processes is well-
known and widely confirmed [4,7,10,11].

Moreover, it needs to be considered that during acoustic cavitation the extremely high local temperature, pressure, and shearing forces – attributable to the collapse of cavitation bubbles and the generation of high-velocity microjets – can also affect both the enzyme molecule and the solid substrate, modifying their chemical and physical properties. Previous studies have shown that intensive ultrasonication can change the protein molecules, resulting in a decrease in the activity of enzymes [12–14].

Additionally, ultrasonic irradiation can also affect the solid cellulosic substrate by disintegrating the fibrous structure and reducing the particle size. Since the properties of solid substrates have a crucial effect on the efficiency of enzyme catalyzed hydrolysis, the particle size reduction caused by cavitation is often considered as one of the possible advantageous effects of power ultrasound. Numerous studies have investigated the ultrasound-aided changes in the particle size of cellulosic and lignocellulosic materials, and the circumstances of ultrasonication, such as the applied type of reactor, intensity, frequency, duty cycle, temperature and its control, duration, solid substrate loading, etc. [15,16]. Nevertheless, the effect of particle size on the enzymatic hydrolysis of cellulosic materials is still contradictory in the scientific literature [17–19], since the morphology, chemical composition, particle size of the original substrate, and the methods used for determining the size of particles (e.g. sieving, microscopy, laser diffraction, etc.) can also affect the final results [20]. Furthermore, in most of the papers, the particle size and its change are usually characterized by an averaged particle size value [21], even though a single number cannot describe the size of a non-spherical particle.

The complex effects of ultrasonication on the particle size of fibrous cellulosic materials and the behavior of disintegrated particles, with varying particle sizes both in the simultaneously or subsequently applied enzymatic digestion, require a more precise description. Ideally, not only a central point of distribution but also one or more values for characterization of the width of the distribution need to be reported. Thus, our research was undertaken to obtain more accurate information (1) about the rate of ultrasound-aided particle size reduction of cellulosic powders and (2) about the mechanism how the particle size reduction occurred can improve the enzymatic hydrolysis of cellulosic materials.

For this, two low-frequency ultrasonic devices (a horn-type reactor and an ultrasonic bath) were used and their effects on the particle size of different cellulosic materials (such as bleached cotton and linen powders) were evaluated. Subsequently, the ultrasonicated powders were subjected to enzymatic hydrolysis by cellulase, and the efficiency of the process was evaluated elucidating the importance of particle size reduction occurred by cavitation. Although the two ultrasonic devices, i.e. the ultrasonic horn and the ultrasonic bath, cannot be compared because the focus of energy and the position of transducers are different. However, the evaluation of these two widely-used ultrasonic devices in the same, well-designed and controlled process is important, since it can contribute to the selection of the right equipment for successful implementation of a specific task.

Thus, in this study, an attempt was made to separate the individual contribution of particle size reduction on the enzyme-aided hydrolysis of solid cellulosic substrates. Fig. 1 presents the key methodologies applied in this study, the powerful techniques (X-ray diffraction /XRD/, particle size analysis /PSA/, scanning electron microscopy (SEM), spectroscopy /UV–VIS/), and the simple tests (water sorption capacity /WSC/, water retention value /WRV/, degree of polymerization /DPv/) used for describing the changes, evaluating the observations and supporting the main statements. The photos of the experimental set-up for ultrasonic and enzyme treatments can be seen in Fig S1 (in Supplementary data).

2. Experimental

2.1. Materials

All chemicals and a commercial acidic cellulase enzyme (Cellulact 1.5 L, 80 FPU/ml, produced by Trichoderma reesei) were purchased from Sigma-Aldrich and used in the experiments. The maximal activity of the enzyme is near 50 °C and at pH 5. 100% bleached cotton and linen plain weave fabrics with a weight per unit area of 157 and 200 g/m², respectively, regarded as cellulose sources and obtained from Pannon-Flax Linen Weaving Co. Győr, Hungary were selected as model substrates for the research. Approximately 1.5 g of the fabrics were ground in a ball-mill (Mixer Mill MM400, Retsch GmbH, Germany) at a frequency of 30 1/s, with 11 stainless steel balls for 2.5 min and the cellulose powders were used in the experiment. The selection of the bleached cotton and linen as cellulose sources can be explained by the high cellulose content and the significant difference in the structure of fibers. While cotton is a single biological cell with a compact structure resistant to disruption to fibrils, linen is a bast fiber with a multicellular fiber structure, where fiber bundles can be easily disintegrated into elementary fibers by physical, chemical and enzyme actions [8,22].

2.2. Ultrasonication and cellulase enzymatic hydrolysis

The powder of both cellulosic fabrics was subjected to ultrasonic and enzymatic treatments. Suspensions of the cellulosic powders with a concentration of 5 g/l and with a total volume of 125 ml were prepared with a 0.05 M acetate buffer (pH 5). Next, the suspensions were thermostated to 50 ± 2 °C and ultrasonicated in a range of 0–60 min either with a horn-type reactor (Sonics & Materials, Model: Vibra-Cell VC505, driving frequency of 20 kHz, power of 500 W, replaceable tip with a

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**Fig. 1.** Schematic representation of the main research processes and introduction of the analytical methods.
diameter of 13 mm), or with an ultrasonic bath (Elmasonic S 40H, 4.5 L, driving frequency of 37 kHz, power of 370 W). The sonicated samples were labelled as US-H-X and US-B-X, respectively, where X shows the duration of sonication in min.

The horn-type reactor was used at 60% amplitude and during sonication the suspensions were also stirred with a magnetic bar at 250 rpm. Control treatments were always carried out simultaneously, with only magnetic stirring but without ultrasonication, and denoted as MS-X. The configuration of the experimental apparatus with the horn-type reactor is presented in detail elsewhere [14].

To gain information about the impact of sonication on the rate of disintegration of solid substrates, the particle size of cellulosic powders was tracked during a 60-minute period and measured at time points 0, 2, 5, 10, 15, 30, 45, and 60 min. Since the ultrasonication with a horn-type reactor was much more efficient than an ultrasonic bath in terms of disintegration of the solid cellulosic particles, in the subsequent experiments the treatment with a horn-type reactor for 15 min (US-H-15) was selected as pretreatment and applied before the enzymatic hydrolysis.

The enzymatic hydrolysis of cellulosic powders was performed under isothermal conditions at 50 °C in a thermostated shaker (Medingen SWB 20, Germany) following the US-H-15 or the control (MS-15) treatments. Cellulase enzyme with concentrations of 3.2, 6.4 and 12.8 FPU/g substrate was added to the ultrasonicated and control cellulose suspensions. Three parallel experiments were performed for 60 min with one sample taken from each flask at each time interval (0, 5, 10, 20, 30, 60 min). Next, the concentration of the reducing sugars (in glucose equivalent) released in the enzyme reaction was measured as described by Miller [23]. Dinitrosalicylic acid served as a stop reagent and the liberated reducing sugars were determined by UV–VIS spectrophotometry at 540 nm with a Unicam 500 UV–Vis spectrophotometer. The reported values are the results of three parallel measurements.

2.3. Particle size analysis

A Horiba Partica LA-950V2 laser diffraction particle size analyzer (Horiba, Japan) was used to define the dimensions of the cellulose particles in the untreated powders as well as the control, ultrasonicated and enzyme-treated suspensions. To adequately describe the particle size of suspensions, the mean (average), as well as median, D50, and D90, for volume distribution values were used, where D50 represents the diameter value, below which 50% of the particles can be found. Further characteristics such as D90 and D10 values were also given. Below D90 and D10, 90% and 10% of the total amount of particles can be found, respectively. In some cases, the width of distribution was characterized by the Span value calculated using the following equation (Eq. (1)):

$$Span = \frac{(D_{90} - D_{10})}{D_{50}} \times 100$$

(1)

2.4. Methods for characterizing the morphology and bulk properties

A Philips PW 1710/PW 1820 X-ray diffractometer was employed at 20 = 4–40° to characterize the structure of bleached cotton and linen fabrics and their powders. The crystallinity indices (CrI%) can be determined by the following equation (Eq. (2)):

$$CrI(\%) = \left(1 - \frac{I_{AM}}{I_{200}}\right) \times 100$$

(2)

where IAM and I200 refer to the intensity of diffraction at 2θ = 18° and the maximum intensity of the 200 lattice diffractions at 2θ = 22.7°, respectively [24].

Images of the original fibrous and ground samples, as well as the ground powders before and after ultrasonication, were taken with a JEOL JSM 6380 LA scanning electron microscope. To induce conductivity, all samples were sputter-coated with gold for analysis.

For estimating the less ordered regions in cellulose, water sorption capacity of the fibrous and ground samples was determined by exposing the triplicate samples, previously dried over P2O5 for 5 days, to an atmosphere of 65% rh at 25 °C for 5 days. CUENE (cupri-ethylene-diamine) fluidity was measured and converted to the viscometric degree of polymerization of cellulose [25]. For characterizing the accessibility of cellulose samples to aqueous reagents, the water retention value (WRV) of fibrous and ground cellulose was determined by soaking the samples in distilled water for 24 h at room temperature and then centrifuging with a Hermlie Z2326 K centrifuge (Labnet, USA) at 5500 rpm for 10 min. The weight of samples was measured right after centrifugation (Wwet) and after drying (Wdry) at 105 °C. The WRV means the weight difference expressed as the percentage of the dry weigh and was calculated with the following equation (Eq. (3)):

$$WRV(\%) = \left[\frac{(W_{wet} - W_{dry})}{W_{dry}}\right] \times 100$$

(3)

3. Results and discussion

3.1. Bulk properties and morphology of the fibrous and ground cellulosic materials

Initially, the original fabrics before and after ball-milling were investigated and characterized with selected physical properties that can have a significant role in enzyme-aided hydrolysis. The difference between the fibrous and ground forms was then quantified. Table 1 presents a summary of these results including data on the particle size, degree of polymerization, crystallinity, and morphology. The latter was introduced by scanning electron microscopy and by measuring the interaction with liquid water and water vapor.

Fig. 2 shows the SEM images of the original fibers from fabrics and the particles of powders. Clearly, even a short ball-milling for 2.5 min can destroy the fibrous structure of both fibers, resulting in cellulose powders with lower crystallinity and degree of polymerization. These changes were accompanied by a significant modification of morphology leading to a considerable increase in water sorption and water retention ability.

As the SEM images of cellulosic powders reveal, besides small particles, some longer fiber fragments remained especially in the linen powder (Fig. 2d). The averaged particle size measured by laser diffraction was 49 μm for cotton, while it was about 123 μm for linen. Ball-milling had only an effect on the length of cotton producing small fiber particles, but did not significantly modify the characteristic appearance i.e. convolutions in the ribbon-like fiber (Fig. 2c). For bast linen, however, the well-known multicellular fiber structure disappeared and the powder consists of flattened elementary fibers and fiber bundles (Fig. 2d). The new morphology and surface properties of ground cotton and linen can be characterized by increased water sorption and water retention as well as lower crystallinity (Table 1). The water sorption capacity, measuring the accessibility of cellulose to water vapor, increased significantly by ball-milling of the bleached cotton and linen (from 6.74 to 7.93% and from 7.09 to 9.14%, respectively) and correlates well with the slightly lower crystallinity of powders (from 83 to 68% for linen).

Table 1

| Cellulosic materials | Particle size (μm) | Water sorption capacity (%) | Water retention value (%) | DPb (%) | CrI (%) |
|----------------------|-------------------|----------------------------|--------------------------|-----|-------|
| Cotton fabric        | –                 | 6.74 ± 0.07                | 45.7 ± 1.7               | 2010 ± 30 | 83 ± 1 |
| Cotton powder        | 49 ± 2            | 7.93 ± 0.05               | 55.9 ± 1.3               | 1130 ± 40 | 81 ± 2 |
| Linen fabric         | –                 | 7.09 ± 0.04               | 48.1 ± 0.6               | 1350 ± 60 | 85 ± 2 |
| Linen powder         | 123 ± 3           | 9.14 ± 0.08               | 68.1 ± 0.2               | 790 ± 20  | 82 ± 1 |

a Mean (average) particle size.
b DPb: Degree of polymerization – viscometric.
to 81% for cotton and from 85 to 82% for linen). Since water molecules cannot penetrate the crystalline regions, both WSC data indicate an increase in the disordered regions of ground cellulose.

The water retention capacity of the fibers depends on the pore structure and surface wetting properties [26]. Since bleached cotton and linen fabrics were used as cellulose sources, and their wettability characterized by water drop test (the method is not introduced) was perfect with a wetting time lower than 1 s, the water retention ability is mainly determined by the pore structure. By destroying the fibrous structure and creating particles with rough surface by ball-milling, the water retention ability of both powders increased significantly compared to the fibrous materials, and the differences in WRV of fibrous and ground cotton and linen were 10.2% and 20.0%, respectively. As the SEM images of the ground samples reveal, the surface of particles – especially for linen – is irregular with cracks and pores capable of retaining water and resulting in the highest WRV (68.1 %) among the samples investigated (Table 1).

Ball-milling extensively degraded the cellulose chains in both bleached cotton and linen, and the loss in DP was 43.7 and 41.5%, respectively. Surprisingly, in a previous study, a significantly lower loss in DP was measured after ball-milling of the raw cotton and linen (14.5 and 30.5%, respectively), and the accompanying increase in water sorption capacity was also more moderate [8] in comparison to the data presented here for the bleached cellulose samples. This indicates that the original, compact structure of raw cotton and linen fibers is more resistant to the destructive effect of ball-milling than that of the bleached fibers with traditional process history including harsh alkaline and oxidative treatments under industrial circumstances.

### 3.2. Ultrasonication of bleached cotton and linen powders either with a horn-type reactor or a sonication bath

Ultrasonication was used as a pretreatment of cellulose suspensions. The experiments aimed (1) to get information about the behavior of cotton and linen particles in aqueous suspension in an ultrasonicated environment, and (2) to evaluate the effect of various types of equipment (ultrasonic horn and bath) on the properties of the cellulose powders. Laser diffraction particle size analysis was the primary test method applied in this study. Besides PSA, changes in morphology induced by ultrasonication were characterized by XRD, and the WRV and WSC values were also measured.

#### 3.2.1. Comparative analysis of particle size and particle size distribution

As discussed in the previous section, the averaged particle size of initial powders used for the experiments was characterized by the mean size, with values for cotton and linen of 49 and 123 µm, respectively (Table 1). The effect of magnetic stirring (control treatment) and ultrasonication of cellulose suspensions either with a sonication bath or a horn-type reactor was investigated. The treatments lasted for 60 min. Samples were taken in triplicates from the cellulose suspensions at various time points for the subsequent laser diffraction PSA. Results in Fig. 3 show the mean size values as a function of the time of treatment.

Clearly, the disintegration of particles is markedly improved in the sonicated environment compared to the treatment with magnetic stirring (MS) alone. In the latter case, only a slight decrease of 15 % was observable for both fibers after 60 min.

Although there was a reduction in the size of particles in both powders, the ultrasonic treatments obviously lead to a stronger decrease in the size of linen particles than that of cotton. The treatment with a horn-type reactor elicited an extremely high shearing force and resulted in a 49 % reduction in mean size, whereas upon applying the ultrasonication bath, a 35 % reduction was observed at the last time point (60 min) for linen particles. For cotton, however, the level of size reduction was more modest with final values of 24% and 16%, respectively. Results, especially in Fig. 3b, clearly show that the particle size of linen decreases abruptly from the beginning of ultrasonication until 15 min, when it reaches almost the final mean size value (US-H-15: 63 µm vs. US-H-60: 61 µm; US-B-15: 86 µm vs. US-B-60: 79 µm). Consequently, after the 15 min time point, the effect of sonication with both reactors was negligible. For cotton, however, a gentler decrease of mean particle...
size led to the final values.

Since a single value cannot describe and characterize the real changes in the size of non-spherical particles such as cellulosic powders, there is a need to deepen the understanding of the particle modifying effect of ultrasonication. Fig. 4 presents the particle size distribution (PSD) curves of both fibers and ultrasonic reactors (US-H and US-B) for 15 and 60 min time points as compared to those of the untreated and only magnetically stirred samples.

Results prove that magnetic stirring even for 60 min was unable to separate and disintegrate the cellulosic particles and did not...
substantially modify the PSD curves of the original cotton and linen powders (red and black curves in Fig. 4, respectively). For cotton, the original, stirred (MS), and sonicated in a bath (UH-B) samples exhibited unimodal and symmetrical PSD curves (Fig. 4 ac) with low Span values ranged from 1.86 to 2.22 (Table S1 in Supplementary data). A more intensive ultrasonication (UH-H) slightly increased the Span to 2.38 (for 15 min), shifting the peaks to smaller particle sizes. The curves remained unimodal but have a perceptible negative skew (Fig. 4a, blue and green curves), indicating the appearance of smaller particles. This is most likely due to the detachment of tiny particles from the surface of fibrous cotton particulates (Fig. 2c), since cotton has a compact structure, which is resistant to further disruption for example by cavitation-induced shearing, or other physical forces [8]. The SEM image of the ultrasonicated cotton particles in Fig. 5a confirms the statements above.

For linen, however, the distribution curve of original particles is three-modal (Fig. 4b,d) with large width of distribution (Span: 2.46), indicating the presence of particles in a broad size range of 5 µm to 500 µm. The two main peaks center at about 100 and 250 µm. After magnetic stirring, the particle size distribution was essentially unchanged but an additional, less pronounced peak in the area of small particles near 15 µm appeared (Fig. 4b,d, black and red curves). Ultrasonication with both devices has a significant effect on the particles of linen powder, resulting in the disappearance of the peak of the largest particles (nearly 250 µm) and enrichment of the smallest particles near 15 µm. The latter was more pronounced with the horn-type reactor even after 15 min treatment (Fig. 4b, blue and green curves). Both changes proved the efficiency of ultrasonication in the disintegration of linen particles, indicating that the share forces generated by microjets are strong enough for separating the multifibrillar linen into elementary fibers with a possible length of 100 µm. The SEM image of linen particles (Fig. 5b) proves the effectiveness of ultrasonication (US-H) on the disintegration of fiber bundles and separation of elementary fibers from the surface.

The shift of the PSD curves to a smaller size and the unambiguous formation of a peak near 15 µm indicate that the ratio of fine particles in the US-H suspension increased significantly (Fig. 4b). The disintegration of the fibrous particles and generation of a large amount of small particles are usually accompanied by the morphological changes of the cellulotic substrate, most likely leading to improved accessibility [27], which can affect the heterogeneous enzyme-aided hydrolysis of cellulose.

3.2.2. Comparative analysis of the morphology of ultrasonicated cellulotic powders

Since even a 15 min-long ultrasonication had a significant particle size reducing and PSD modifying effect on the cellulotic powders as discussed previously, in the following experiments the changes in the morphology solely of samples treated for 15 min were investigated. As Fig. 6 shows the interaction of cellulotic particles with liquid water (expressed in WRV) has changed significantly, especially for samples treated with a horn-type reactor. Results proved that linen powder ultrasonicated with a horn-type reactor (US-H-15) has a markedly improved water retention value of 85.8%, whereas the WRV of cotton powder is more modest with values of around 59%. The WRVs for MS and US-B samples are significantly lower: the values for cotton are 51.6 and 53.7%, and for linen are 67.9 and 69.8%, respectively. Similarly, a significant increase in water holding capacity of okara fibers occurred by ultrasonication with increasing power density [28].

Since water retention power is a measure of the accessibility of fibers to aqueous baths during wet processing [26], it can predict the effectiveness of ultrasonication (with a horn-type reactor, US-H) as a pretreatment in the cellulase enzymatic hydrolysis of cellulotic powders. In that case, the shear forces generated in the ultrasonicated suspension were much stronger than those caused by a moderate magnetic stirring or an ultrasonic bath, and they were able to separate solid particles stuck together in cotton powder or elementary fibers in fiber bundles of linen. The new surfaces contain irregularities, cavities, and external fibrils, that contribute significantly to the increased water holding capacity of the ultrasonicated substrates. It was proven for softwood kraft pulp fibers [27] that not only pores but also external fibrils formed under high shear forces and present on the surface of fibers can retain a significant amount of water. In this study, the surface fibrillation of linen particles by ultrasonication (US-H) was very pronounced (Fig. 5b) that can contribute to the water holding capacity and explain the large value of 85.5% (Fig. 6b).

The slight changes occurred by magnetic stirring and ultrasonication were measured as the water vapor sorption of cellulotic samples (Fig. 6). The values for cotton and linen are in a narrow range of 7.76–7.92% and 8.13–9.11%, respectively. Clearly, the applied treatments have only a negligible effect on the free hydroxyl groups in the amorphous regions and at the surface of crystallites of cellulose that are responsible for water vapor sorption. The data correlate well with the crystallinity (Table S1 and Fig. S2 in Supplementary data), which remained almost unchanged during the processes (CrI: 79–82%). Shearing treatment of bleached cellulose with homogenization resulted in an increase of water retention, but the crystallinity of cellulose remained unchanged [29].

Furthermore, all samples were dried after the applied treatments since both the water retention and water sorption tests require dry cellulotic powders. Since drying causes an irreversible collapse of pores and formation of interfiber hydrogen bonds (Kimura et al., 2020), the test methods likely under-estimate the accessibility. Thus, a comparison of values in Fig. 6 with those of the untreated powders (Table 1, WSC: 7.93 ± 0.05% and 9.14 ± 0.08%; WRV: 55.9 ± 1.3 and 68.1 ± 0.2%, for cotton and linen, respectively) is superfluous.

It is clear that in the present experiment, low-frequency ultrasonic pretreatment with a horn-type reactor was more efficient and resulted in more favorable changes in substrate properties than the ultrasonic bath, presumably facilitating a more efficient enzymatic treatment of the cellulose powder. However, it should also be noted that in other processes, e.g. in enzyme-assisted bleaching of cotton, the most favorable results, either in terms of product properties or process parameters, can
be achieved by combining and simultaneous using different ultrasonic devices, which also have different frequencies, geometry and performance [30].

3.3. Enzymatic hydrolysis of ultrasonicated cotton and linen powders

3.3.1. Reducing sugar liberation

Ultrasonication with a horn-type reactor was proven to be the most effective pretreatment by modifying the particle size and particle size distribution as well as the structural and bulk properties of cotton and linen powders. Since the most significant changes in the particle size occurred within 15 min and beyond only minor variations were detected, ultrasonication with a horn-type reactor for 15 min was selected as pretreatment and applied prior to the cellulase enzymatic digestion. As a control, a pretreatment under magnetic stirring for 15 min was applied. The enzymatic hydrolysis was performed under horizontal shaking.

Consistent with the structural changes and particle size reduction, an increase in enzymatic digestion of the ultrasonicated powders was anticipated. As the results in Fig. 7 reveal, a significant increase can be observed in the rate of hydrolysis with respect to that of the control cellulose. Besides, the concentration of reducing sugars liberated in hydrolysis seems to be directly related to the enzyme concentration.

The comparison of reducing sugar concentration data at the 60 min time point (Table S2 in Supplementary data) reveals that the substrate modifying effect of ultrasonication, such as particle size reduction and an increase in accessibility, contributed significantly to the efficiency of the subsequently applied enzymatic hydrolysis and resulted in an increase of the hydrolysis products. The difference in reducing sugar concentrations between the ultrasonicated and control samples at different enzyme loads is in the range of 12–27% and 13–39% for cotton and linen, respectively, which can exclusively be attributable to the advantageous interaction between the solid substrate and ultrasonication. It was also proven that at smaller particle sizes (33–37 µm) of the cellulosic substrate (such as oak sawdust), higher rate of enzymatic digestion was measured, and after 72 h hydrolysis, and approximately 50 % increase in glucose concentration was detected [31]. However, further decrease in particle size to less than 1 µm did not increase the concentration of reducing sugars and the rate of hydrolysis [30]. For microcrystalline cellulose pretreated with homogenization, the increased WRV of the substrate correlated to the enzymatic hydrolysis rate [29].

Additionally, at the 15 min time point (Fig. 7, Table S2 in Supplementary data) the difference is even more significant, as the increase has almost reached 50% and 80% for cotton and linen, respectively, with an enzyme load of 12.8 FPU/g cellulose. These data can provide an opportunity to estimate separately the pure and undoubtedly positive contribution of ultrasonication (15 min) to the heterogeneous enzymatic hydrolysis (15 min) which is based exclusively on the substrate modifying effect of ultrasonication (i.e. particle size reduction and changes in morphology). Otherwise, in ultrasound-aided enzymatic hydrolysis (e.g. for 15 min) not only the positive i.e. the substrate modifying (see above) and mass transfer accelerating effects of ultrasonication prevailed, but also the negative impact, such as a reduction in enzyme activity [4,13,14]. Notwithstanding that several advantageous and
disadvantageous processes take place simultaneously in the heterogeneous enzymatic hydrolysis performed in an ultrasonicated environment, the outcome is predominantly positive.

### 3.3.2. Changes in particle size during the enzymatic hydrolysis

Since the most effective hydrolysis occurred at the highest enzyme load (12.8 FPU/g cellulose, Fig. 7), this process was selected and further investigated to identify the effect of enzyme action on the size and size distribution of particles. Fig. 8a and b show the PSD curves of cotton and linen powders, respectively, before and after the enzymatic hydrolysis. Results prove that (1) the cellulase enzyme treatment affects the particles and changes their average size and PSD, (2) the changes of particles during the enzymatic digestion are determined by the pretreatment (i.e. ultrasonication or magnetic stirring).

Clearly, a small shift of the PSD curves took place. Interestingly, the direction of the shift depends on the type of pretreatments: for ultrasonicated samples, the curves shifted to the right — toward the larger particles; for the control pretreatment, however, the curves shifted to the left — to smaller particle sizes – by the enzymatic treatment.

The enzyme-treated cotton (Fig. 8a) has a slightly narrower particle size distribution than the initial ultrasonicated sample, indicating the removal of the smallest particles by enzymatic digestion. The shape of the PSD curves of the control sample (MS-15), however, remained unaffected by the enzymatic treatment.

The PSD-modifying effect of enzymatic treatment is particularly pronounced in linen powders (Fig. 8b). For the ultrasonicated sample, similar to the cotton counterpart, the number of small particles with the average size of 15 µm slightly decreased, while a less pronounced peak in the region of large particles near 250 µm appeared. This peak with a significantly higher frequency can also be found in the PSD curve of the MS-15 control and MS-15-E60 samples, but in the latter case, due to the enzyme action, the ratio of large particles decreased significantly.

A plausible explanation for the increase of average particle size and the shift of PSD curves toward the higher values in the US-H-15-E60 process can be the fast enzymatic digestion and disappearance of the enzyme action, the ratio of large particles decreased significantly. Since the ultrasonicated suspension is rich in small particles, their disappearance or wane by enzymatic digestion overcomes the production of particles with the larger size. This would explain the shift of the PSD curve of the US-H-15-E60 suspensions for both fibers to the direction of small particle sizes. The control pretreatment, however, did not affect significantly the particle size and PSD, and did not alter the ratio of small particles. Thus, in the control (MS-15-E60) treatment the disintegrating effect of cellulase is the predominant process, contributing to the shift of PSD curves to the right, toward the larger particles.

### 4. Conclusions

In this research bleached cotton and linen in ground form served as cellulose sources to evaluate (1) the rate of the ultrasound-aided particle size reduction and the accompanying changes in the morphology of cellulose powders and also (2) the effect of size reduction on the efficiency of the subsequent cellulase enzymatic hydrolysis. Results clearly

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**Table 2**

Changes in particle size of cotton and linen powders during a cellulase enzymatic hydrolysis with an enzyme load of 12.8 FPU/g cellulose and for 60 min (E60). The effect of pretreatments such as ultrasonication with a horn-type reactor (US-H-15) or magnetic stirring for 15 min (MS-15, control) on the mean size and $D_{10}$, $D_{50}$ and $D_{90}$ characteristics.

| Cellulosic materials | Treatments        | Mean size | $D_{10}$ | $D_{50}$ | $D_{90}$ |
|----------------------|-------------------|----------|---------|---------|---------|
|                      |                   | (µm)     |         |         |         |
| Cotton               | MS-15             | 49.9 ± 2.5 | 14.6 ± 0.1 | 38.1 ± 0.1 | 98.8 ± 5.5 |
|                      | MS-15-E60         | 48.4 ± 1.3 | 14.4 ± 0.1 | 34.4 ± 0.3 | 89.5 ± 4.5 |
|                      | US-H-15           | 40.7 ± 0.3 | 8.2 ± 0.1 | 30.0 ± 0.2 | 79.5 ± 1.2 |
|                      | US-H-15-E60       | 47.1 ± 0.8 | 13.7 ± 0.1 | 31.9 ± 0.3 | 95.6 ± 1.4 |
| Linen                | MS-15             | 110.2 ± 0.3 | 26.8 ± 2.6 | 95.5 ± 3.4 | 258.0 ± 2.8 |
|                      | MS-15-E60         | 81.4 ± 0.9 | 17.3 ± 0.2 | 59.7 ± 0.5 | 182.1 ± 6.0 |
|                      | US-H-15           | 62.9 ± 0.9 | 13.2 ± 0.1 | 47.5 ± 0.3 | 121.5 ± 3.6 |
|                      | US-H-15-E60       | 66.3 ± 1.1 | 14.3 ± 0.2 | 47.6 ± 0.5 | 144.3 ± 6.1 |

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*Fig. 8. The effect of pretreatments (US-H-15 or MS-15) on the particle size distribution of residual cotton (a) and linen (b) particles measured after the cellulase enzymatic digestion for 60 min (E60) at 12.8 FPU/g cellulase enzyme load.*
proved that magnetic stirring (control) only marginally affected the structural properties of cellulotic powders and the influence of the ultrasonic bath was also moderate. Treatment with a horn-type reactor, however, generated strong waves and microjets, leading to the significant modification of the particles. The extremely strong shear forces resulted by the treatment led to reduction of the average particle size, a change in the particle size distribution and an increased accessibility e.g. due to surface fibrillation. Additionally, a more severe effect of ultrasonication was observed on linen than on cotton.

The presence and amount of particles with a smaller particle size (approx. 15 μm for linen) and their increased accessibility showed a major effect on the rate and efficiency of the subsequent enzymatic hydrolysis. The enzyme preferably digested the small particles generated by ultrasonication and resulted in a high concentration of reducing sugars. Furthermore, the enzyme action was able to disintegrate the larger particles. The disappearance of the smaller and appearance of the larger particles by the enzyme action resulted in a modified PSD as the new size distribution curves shifted to the right towards the larger average particle size regions. In the control suspension, however, the number of small particles was negligible, and the enzyme disintegrating effect was dominant.

Clearly, in ultrasound-aided enzymatic hydrolysis the applied ultrasonication continuously provides small and highly accessible solid particles for the enzymatic digestion, promoting the enzyme action and contributing to the high overall yield of the hydrolysis. Based on the data obtained from the separated ‘ultrasonic pretreatment – enzymatic hydrolysis’ system introduced above, it can be concluded that the particle size reducing and accessibility increasing effects of ultrasonication on the solid particles could even result in a 50% and 80% increase in reducing sugar concentration for cotton and linen, respectively (Table S2 in Supplementary data, at 15 min time point).

References

[1] P.B. Subhedar, P.R. Gogate, Intensification of enzymatic hydrolysis of lignocellulose using ultrasound for efficient bioethanol production: a review, Ind. Eng. Chem. Res. 52 (2013) 11816–11828.
[2] Z.M.A. Bundhoo, R. Mohee, Ultrasound-assisted biological conversion of biomass and waste materials to biofuels: a review, Ultrason. Sonochem. 40 (2018) 298–313.
[3] A. Kadlic, B. Palmqvist, G. Liden, Effects of agitation on particle-size distribution and enzymatic hydrolysis of pretreated spruce and giant reed, Biotechnol. Biofuels 7 (2014) 77.