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# Short-time ultrasonication treatment in enzymatic hydrolysis of biomass

**Abstract:** To improve the conversion of enzymatic hydrolysis of biomass in an energy-efficient manner, two short-time ultrasonication strategies were applied on six types of biomass with different structures and components. The strategies include pre-sonication before the hydrolysis and intermittent sonication during the ongoing hydrolysis. The microstructures of each type of biomass were characterized by scanning electron microscopy to investigate the potential correlation between biomass structure and ultrasonicaton. The concentration of resultant reducing sugar was measured to evaluate the efficiency of the hydrolysis. The results indicate that hydrolysis efficiency greatly depends on the initial structures of biomass and that short-time ultrasonication can yield up to 27.5% improvement in hydrolysis efficiency with only 120 s of sonication.

**Keywords:** enzymatic hydrolysis, lignocellulose, reducing sugar, ultrasonication

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## Introduction

Biofuels obtained within the scope of the biorefinery concept are a promising alternative to fossil fuels. Biorefinery means an effective conversion of grass, wood or straw to biofuels, e.g., to ethanol or butanol – as known from the traditional fossil fuel refinery (Bozell and Petersen 2010; Dautzenberg et al. 2011; Hörhammer et al. 2011).

Cellulose can be converted to fuel-grade ethanol by enzyme-catalyzed hydrolysis and subsequent fermentation (Kumar et al. 2009b). However, the efficiency of enzymatic hydrolysis is often low because the partly crystalline nature of cellulose and the complex structure of other polymers surrounding cellulose hinder the accessibility of cellulase to the substrate (Kumar et al. 2009a). The hydrolytic efficiency can be elevated by pretreatments with diluted acids, steam explosion, and an organosolv treatment, in the course of which the supramocelular structrure of the cell walls is destroyed to varying degrees (Mosier et al. 2005; Mendes et al. 2009; Zheng et al. 2009; García et al. 2011; Kirsch et al. 2011; López et al. 2011; Rodríguez-López et al. 2012; Vila et al. 2012). For instance, treatment with *N*-methylmorpholine N-oxide (NMMO) and ionic liquids can remarkably improve the hydrolytic efficiency of lignocelluloses (Dadi et al. 2006; Liu et al. 2011). But physical pretreatments by low-pressure steam, high-power ultrasonication, and continuous extrusion are also effective in this regard (Lamptey et al. 1985; Lee et al. 2009; Schütt et al. 2011). The combination of ultrasonication with other pretreatments is also promising (Imai et al. 2004). Previous reports were focused mainly on a long-term or continuous sonication process prior to the hydrolysis; the effects of short-time or intermittent sonication processes have been neglected so far.

In the present paper the effects of short-time sonication will be investigated in terms of the hydrolysis efficiency by means of two strategies: (1) pre-sonication before the hydrolysis and (2) intermittent sonication throughout the hydrolysis. Six biomass specimens from different sources will serve as substrate, namely: pure cellulose, *N*-methylmorpholine-N-oxide treated cellulose, native red oak and switchgrass, and solvent-extracted red oak and switchgrass. The morphological difference of the above biomasses will be characterized by scanning electron microscopy (SEM). The treatment effects will also be detected by determination of the reducing sugar (RS) content in the hydrolysates.

## Materials and methods

### Materials

*N*-methylmorpholine N-oxide (NMMO, Alfa Aesar, Ward Hill, MA, USA) was concentrated to approximately 83% (w/w) before use. Pure cellulose (PC) (with average particle size of 20 μm×900 μm) (TC2500, ≥99.5% alpha cellulose) was provided by CreaFill Fibers Corp. (Chestertown, MD, USA). NMMO-treatment: PC (5.0 g) was stirred vigorously with NMMO (100 ml) at 120°C for 30 min; then the hot cellulose solution

was immediately poured into 500 ml of boiling water under vigorous stirring. The cellulose precipitate was collected by filtration and then washed with water 10 times to remove the residual NMMO (200 ml water for each time). The resultant $PC_{NMMO}$ was kept at 4°C.

Flakes (0.5 cm×1 cm) of southern red oak (*Quercus falcate*, RO, harvested in Knoxville, TN, USA) were air-dried. Dried switchgrass stems, grown during the 2008 growing season at UT Research Milan Research and Education Center (Milan, TN, USA) and harvested in January 2009, were cut into small pieces of about 1.0 cm in length. The organosolv-treated switchgrass ($SG_{org}$) and red oak ($RO_{org}$) were prepared by an organosolv process based on the digestion with ethanol/water containing $H_2SO_4$ and methyl isobutyl ketone (MIBK, Alfa Aesar, Ward Hill, MA, USA), (0.1 M in concentration, 160°C for 56 min, according to Bozell et al. 2011). After digestion the biomass was allowed to cool to room temperature and then washed with distilled water. Kappa numbers were determined without drying (TAPPI Test Methods, T236 cm-85, Tappi Press, 1996). The pulp yields were determined after drying. Average Kappa number was 35.6 for $RO_{org}$ and 27 for $SG_{org}$. Literature data indicate 23% lignin content for RO (White 1987) and 17% for SG (Keshwani and Cheng 2009). The lignin contents of these extracted materials were 4.6% for $RO_{org}$ and 3.5% for $SG_{org}$, based on recalculation of the Kappa numbers with a factor of 0.129 (TAPPI Test Methods, T236 cm-85, Tappi Press, 1996). Accordingly, 18.4% and 13.5% lignin were removed from RO and SG after the organosolv treatment, respectively. For abbreviations see Table 1.

Spezyme CP cellulase (product code A03117) was kindly provided by Genencor International (Rochester, NY, USA), with an activity of 50 FPU $ml^{-1}$ that was determined according to Adney and Baker (1996). The method is based on "filter-paper units" (FPU $ml^{-1}$) of the undiluted (original) enzyme solution.

Ultrasonication was carried out with a high-intensity (1500 W) Sonics Ultrasonicator (Sonics & Materials Inc., Newtown, CT, USA) equipped with a VCF 1500 ultrasonic processor and a booster at room temperature (without cooling). The power output was always 80% of the maximum. Scanning electron microscopy (SEM) images were collected with a Zeiss LEO 1525 microscope (LEO Electron Microscopy Inc., Thornwood, NY, USA).

## X-ray diffraction (XRD) of PC and $PC_{NMMO}$

Instrument: X'Pert Pro diffraction system (Panalytical Incorporated, Westborough, MA, USA). The vacuum-dried sample was placed on a glass plate fixed on the MDR cradle. The diffraction spectrum was taken at 2.4° from 2θ=10–30° with a step size of 0.02°. Cu-K$\alpha$ radiation (λ=1.5418 Å) was generated at 45 kV and 40 mA. The crystallinity index ($C_I$) was calculated according to Nelson and O'Connor (1964): $C_I$ (%)=100 ($I_{cr} - I_{am}$)/$I_{cr}$, where $I_{cr}$ is the diffraction intensity of peak at 2θ≈22.6° for cellulose I and 21.7° for cellulose II, and where $I_{am}$ is the intensity of the amorphous background (2θ≈19° for cellulose I and 16° for cellulose II).

## Effects of ultrasonication

Four PC specimens were prepared in a sodium acetate/acetic acid buffer (pH=5) with the same loading mass (0.4 g solid PC in 25 ml buffer solution); 0.5 ml Spezyme was introduced into each solution and ultrasonication was applied for 0, 10, 20 and 30 s, respectively, without cooling and air-exclusion. Then the samples were placed in a water-bath shaker at 50°C and 180 rpm for enzymatic hydrolysis. The activity of the sonicated enzyme was determined by monitoring the RS concentration of the resulting solution over 24 h by the BCA method (Zhang and Lynd 2005).

## Enzymatic hydrolysis supported by ultrasonication

Three specimens with the same loading mass (0.4 g solid equivalent) were introduced into 25 ml sodium acetate/acetic acid buffer (pH=5). The first specimen was added with 0.5 ml of Spezyme and then put into a water-bath shaker at 50°C and 180 rpm as a control. The second specimen was treated with 120 s ultrasonication before the enzyme was added (pre-sonication strategy, PS). The third one was treated with six repetitions of periodic 20 s ultrasonication at 0, 3, 8, 24, 48 and 72 h during the ongoing hydrolysis (total sonification time 120 s) (intermittent sonication strategy, IS). The RS concentration was determined after a certain time interval. Three replications were carried out for each treatment condition and the conversion was determined by the BCA method. For abbreviations see Table 1.

# Results and discussion

## X-ray diffraction

The effect of NMMO treatment on pure cellulose (PC) was investigated by XRD (Figure 1). The diffraction intensity

**Table 1** Materials and their abbreviations in text and in the figures.

| Material | Abbreviation | Kappa no. | Lignin content (%) | Abbreviations after 120 s of sonication | |
|---|---|---|---|---|---|
| | | | | Pre-sonication (PS) | Intermittent sonication (IS) |
| Pure cellulose | PC | – | – | $PC_{PS}$ | $PC_{IS}$ |
| PC, NMMO treated | $PC_{NMMO}$ | – | – | $PC_{NMMO, PS}$ | $PC_{NMMO, IS}$ |
| Southern red oak | RO | – | 23[a] | $RO_{PS}$ | $RO_{IS}$ |
| RO, organosolv treated | $RO_{org}$ | 35.6 | 4.6 | $RO_{org, PS}$ | $RO_{org, IS}$ |
| Alamo switchgrass | SG | – | 17[b] | $SG_{PS}$ | $SG_{IS}$ |
| SG, organosolv treated | $SG_{org}$ | 27 | 3.5 | $SG_{org, PS}$ | $SG_{org, IS}$ |

According to White (1987)[a] and Keshwani and Cheng (2009)[b].

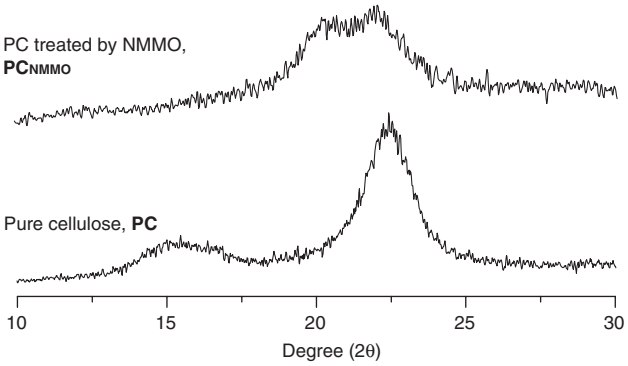


**Figure 1** X-ray diffraction diagrams of pure cellulose (PC) and NMMO-treated PC ($PC_{NMMO}$).

of peak at 2θ≈22.6° was assigned to cellulose I, and 2θ≈19° to the amorphous background. For $PC_{NMMO}$, the peak at 2θ≈21.7° was supposed to be cellulose II, and 2θ≈16° is the amorphous background. It was verified that the starting PC was mostly crystalline cellulose I (Moon et al. 2011), with a calculated $C_I$ of 85.8%. The $PC_{NMMO}$ sample exhibited a typical XRD spectrum of crystalline cellulose II, but with a lower $C_I$ of 67.8%. This indicated that the PC (cellulose I) undergoes a complete dissolution, which was followed by recrystallization during precipitation, in the course of which a crystalline form arose being a combination of cellulose I and cellulose II in case of $PC_{NMMO}$ (Moon et al. 2011).

## Observation of the treatment effects by SEM

Photographs and SEM images of PC, $PC_{NMMO}$, RO, SG, $RO_{org}$, and $SG_{org}$ are presented in Figure 2. According to Figure 2a–f, PC was solid fiber-like material with an approximate 20 μm diameter and a length of hundreds of microns, while the $PC_{NMMO}$ was a homogeneous cream-like pulp and its size was too small for a microscopic measurement.

The images of RO and $RO_{org}$ (Figure 2g–l) are very similar but the SEM images show that RO is highly compacted and its small fragments and fibers cannot be distinguished, while $RO_{org}$ displays extensive fibrillation with flexibly entangled microfibers. This observation can be interpreted as a result of delignification during the organosolv process with lignin removals of 18.4% (RO) and 13.5% (SG). According to Ibrahim and Glasser (1999), the microcrystalline cellulose remains after such a treatment intact. The structure of RO was like solid rods with hundreds of μm in diameter and thousands of μm in length. The $RO_{org}$ comprised loose fibers in a bundle-like structure with an approximate of 10 μm diameter and a length of hundreds of μm.

Images for SG and $SG_{org}$ are presented in Figure 2m–r, respectively. The SEM images revealed that the SG retained its structural uniformity and it was flaky rather than solid rod-like. SG flakes are circa hundreds of μm in width, thousands of μm in length, and tens of μm in thickness. In the $SG_{org}$, the big SG flakes are fragmented into small flexible fibers with thousands of μm in length and are circa 10 μm in diameter. In appearance the $SG_{org}$ was similar to the PC.

## Effects of ultrasonication on enzyme activity

First, a series of Spezyme containing PC samples were treated in different sonication periods, and then the samples were hydrolyzed and the RS concentration was determined after 24 h. The RS concentration without sonication was 2.8 mg $ml^{-1}$, and the concentrations after sonication of 10, 20, and 30 s were 2.6, 2.6 and 2.7 mg $ml^{-1}$, respectively. Accordingly, short-time ultrasonication did not significantly change the activity of Spezyme in the system. Therefore the conditions for the following hydrolysis experiments were designed either as a one-time 120-s sonication (pre-sonication strategy, PS) or six repetitions of a periodic 20-s sonication (intermittent sonication strategy, IS, 120 s in total).

## Enzymatic hydrolysis of cellulose and NMMO-treated cellulose

The results of hydrolysis time vs. RS concentration for PC and $PC_{NMMO}$ are presented in Figure 3a. Overall the RS yield of $PC_{NMMO}$ was almost twice that of PC after 96 h. This was due to the crystallinity reduction of $PC_{NMMO}$ and its improved accessibility for the enzymes (Kuo and Lee 2009). For both PC and $PC_{NMMO}$, when hydrolysis was promoted by either PS or IS approach, a very tiny difference in RS yield was observable. This means that the responsive effect of PC and $PC_{NMMO}$ was poor towards the short-time ultrasonication. For the PC, as a pure cellulose with high degree of crystallinity, the energy of the short-time ultrasonic treatment was not sufficient for lowering the crystallinity. The situation was similar for $PC_{NMMO}$, as its crystallinity was already diminished and its particle size was small enough, so that sonication could not improve further the accessibility of enzymes.

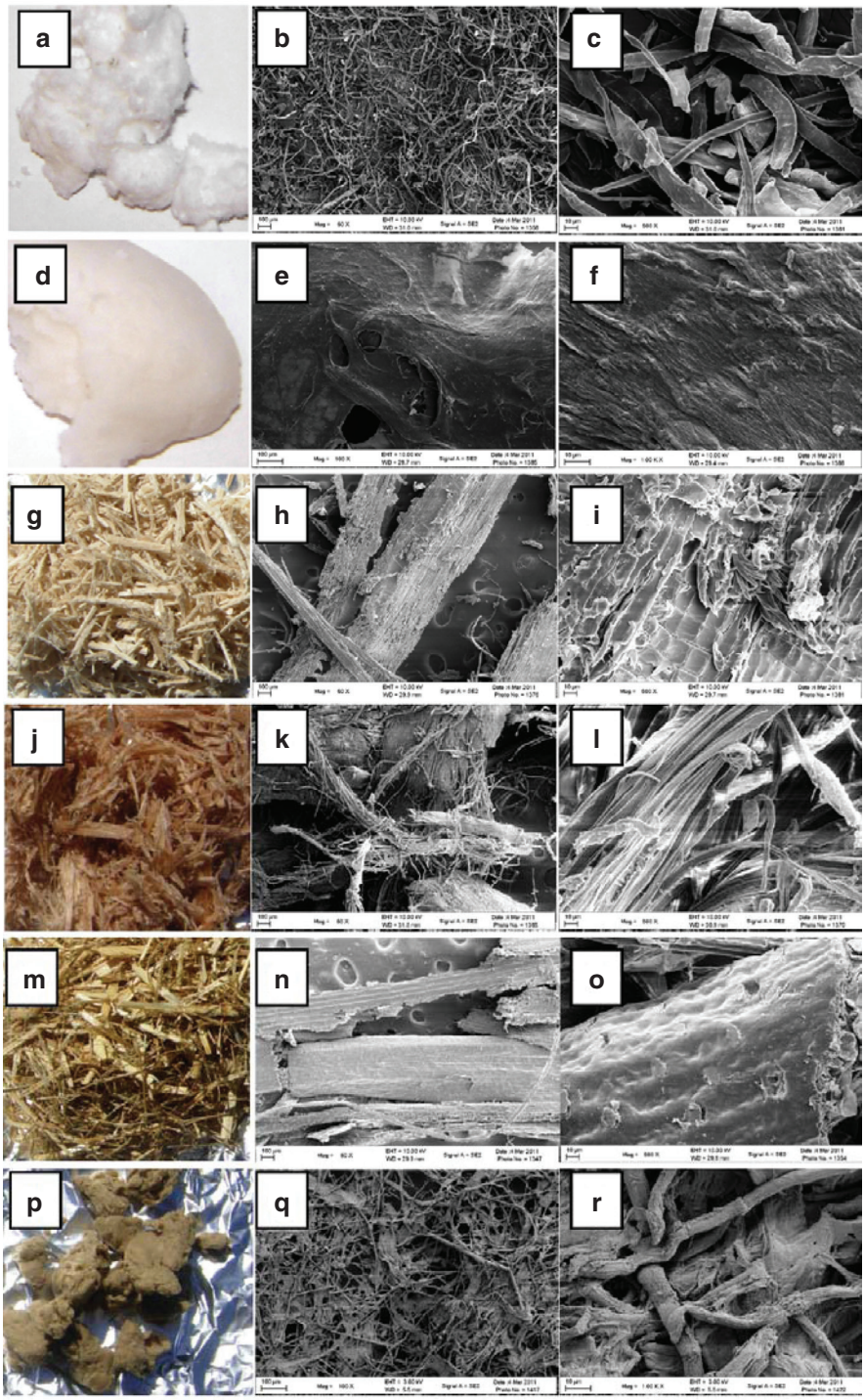


**Figure 2** Photos and SEM images of lignocellulosic materials: Pure cellulose (PC) fibers (a, b, c); NMMO-treated ($PC_{NMMO}$) (d, e, f); Pristine red oak, RO (g, h, i); Organosolv treated RO ($RO_{org}$) (j, k, l); Virgin switchgrass (SG) (m, n, o); Organosolv treated SG ($SG_{org}$) (p, q, r).

## Enzymatic hydrolysis of red oak and organosolv-treated red oak

The results of hydrolysis time vs. RS concentration for RO and $RO_{org}$ are shown in Figure 3b. The resultant average RS concentration of RO was 0.45 (control), 0.48 (120 s PS) and 0.40 mg $ml^{-1}$ (120 h IS). The RS yields of RO were much lower than that of pure cellulose (PC), probably because of the masking effect of the high lignin content in RO. The pristine RO treated with 120-s

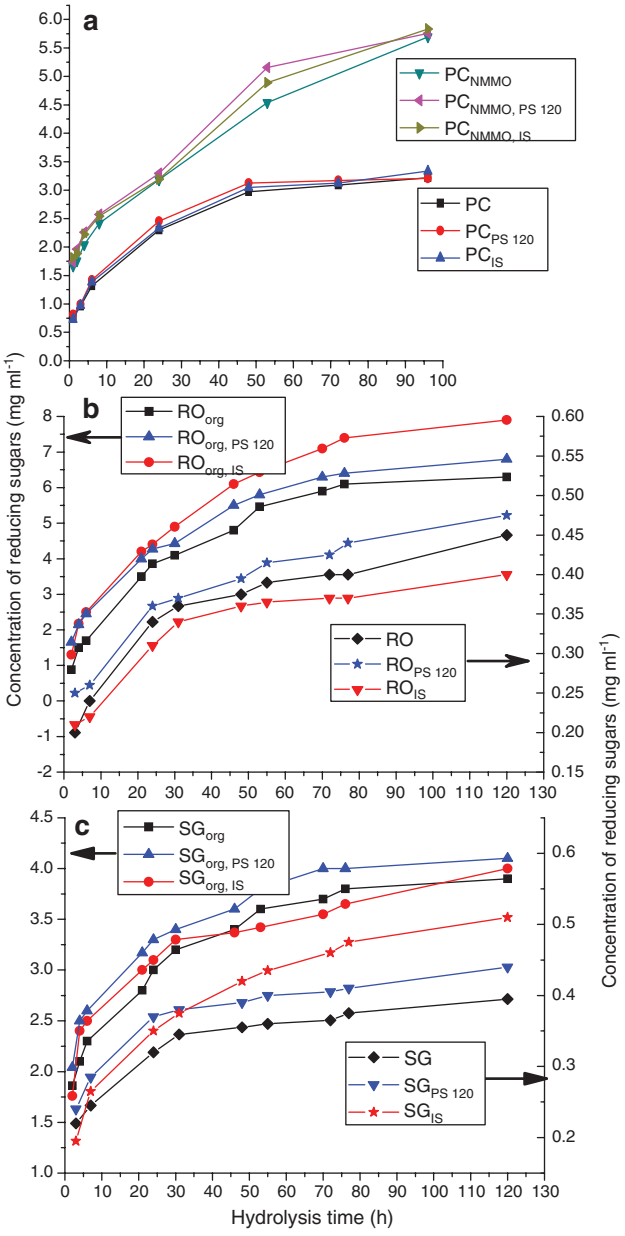


**Figure 3** Plots of "reducing sugar yields vs. hydrolysis time" as a function of ultrasonic treatment. For abbreviations see Table 1. Numbers in lower case refer to sonication time in seconds. a) Plots for pure cellulose (PC) and NMMO treated PC ($PC_{NMMO}$); b) Plots for red oak (RO) and organosolv treated RO ($RO_{org}$); c) Plots for switchgrass (SG) and organosolv treated SG ($SG_{org}$).

PS had a 6.7% RS increment [(0.48–0.45)/0.45=6.7%] compared to the control. The sample treated with the IS strategy had approximated 11.1% RS decrement [(0.40–0.45)/0.45=11.1%] compared to the control. The reason for the RS decrement in case of the IS strategy can be assumed: the lignin content of RO is around 23% (White 1987) and 4.6% in $RO_{org}$, thus the high lignin content may be the reason for its low RS content. That lignin deactivates the enzyme is well known (Liao et al. 2005; Mussatto et al. 2008). It is reasonable to conclude that the IS strategy may allow more lignin release into the solution because the supramolecular structure of the cell wall can be gradually eroded during the periodic sonication. This is not the case for a simple sonication before the hydrolysis (PS strategy).

It is also visible in Figure 3b, that the $RO_{org}$ and $RO_{org, PS120}$ specimens had very similar RS yields (with ~8% difference) after 120 h. But the $RO_{org}$ treated with the IS strategy ($RO_{org, IS}$) showed distinct elevated RS yield after 120 h. The average RS concentration of $RO_{org}$ was 6.3 mg $ml^{-1}$, while that of $RO_{org, IS}$ was 7.8 mg $ml^{-1}$, so the RS increase was 23.8% [(7.8–6.3)/6.3=23.8%]. Here the loose fiber bundle-like structure of $RO_{org}$ was believed to be highly responsive to ultrasonication because the loose bundle structure should have been destroyed more easily by ultrasonication than the compact and rod-like structure in pristine RO.

## Enzymatic hydrolysis of switchgrass and organosolv-treated switchgrass

The plots hydrolysis time versus RS yield for SG and $SG_{org}$ are presented in Figure 3c. The resultant average RS yields of SG are 0.40 (control), 0.44 (120-s PS strategy), and 0.51 mg $ml^{-1}$ (IS strategy). Switchgrass subjected to PS sonication shows a 10% RS increment [(0.44–0.40)/0.40=10%] compared to the control, while the IS strategy resulted in a 27.5% RS yield improvement [(0.51–0.40)/0.40=27.5%]. Accordingly, the IS strategy can remarkably improve the efficiency of enzymatic hydrolysis of SG.

For $SG_{org}$ (Figure 3c) the RS yields were 3.8 (control), 4.1 (120-s PS strategy), and 3.9 mg $ml^{-1}$ (IS strategy). Thus the results are very similar irrespective of the sonication. The interpretation based on the morphology of $SG_{org}$ was that the fibers were very thin and flexible (with an approximate 10 μm diameter), so that the additional short-time sonication did not contribute significantly to their further size reduction and the improvement of the enzymatic accessibility.

Therefore, as shown in Figure 2n and 2o, the SG with the dimensions of 10×100×1000 $\mu m^3$ has a very good response to short-time sonication. $SG_{org}$ (Figure 2q and 2r), on the other hand, with dimensions of 10 μm in diameter and thousands of μm in length already have favorable properties, which could not be improved further by short-time sonication.

## Conclusions

The initial structure and composition of biomasses significantly affect the efficiency of enzyme-assisted hydrolysis. The organosolv delignification improved the biomass conversion with a factor of circa 10. The effect of a 120-s pre-sonication was not pronounced. The enzymatic accessibility of materials with compact and bulk structures (like untreated PC and RO) cannot be improved further by short-time ultrasonication. The improvement of materials with small particle size (like $PC_{NMMO}$ and $SG_{org}$) was less than 10%. The intermittent sonication strategy showed a remarkable improvement (>20%) in enzymatic hydrolysis for some specimens after a total treatment time of 120 s. The short-time sonication was promising in the case of plant materials with loosely fiberized or flaky structure and moderate size like $RO_{org}$ and SG.

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