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The effect of the time process of enzymatic hydrolysis on nanocellulose properties

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Abstract: *The effect of the time process of enzymatic hydrolysis on nanocellulose properties* - the aim of the study was to evaluate the effect of enzymatic hydrolysis time on the properties of obtained nanocellulose. Two cellulose materials were tested as a raw material for nanocellulose production in the experiment: Avicel and Whatman. The cellulolytic enzyme obtained from the fungus Trichoderma reesei was used to carry out the enzymatic hydrolysis reaction. Enzymatic hydrolysis was performed on cellulose using the reaction times of 0.5, 1, 2 and 4 hours. In order to characterize the obtained materials, the following analyses were used: infrared spectroscopy, X-ray diffraction and dynamic light scattering. The recorded results showed that cellulose after enzymatic hydrolysis showed similar parameters (particle size, XRD patterns and degree of crystallinity) after all the applied reaction times.

Keywords: nanocellulose, enzymatic hydrolysis, XRD, FTIR, DLS

INTRODUCTION

The enzymatic hydrolysis of cellulose is based on breaking β-1,4-glycosidic bonds with the participation of cellulases, which are cellulolytic enzymes [Barzkar and Sohail 2020, Thapa et al. 2020]. Cellulases are mainly produced by some fungi of such genera as *Trichoderma*, *Aspergillus*, *Schizophyllum* and *Penicillium* as well as bacteria of several genera, e.g. *Clostridium*, *Cellulomonas*, *Bacillus* or *Thermomonospora* [Leja et al. 2009, Xiao et al. 2020].

Cellulolytic enzymes can be divided depending on their activity on endoglucanases (breaking down long cellulose chains), exoglucanases (reacting with external groups of the sugar molecule) and β-glucosidases (breaking down the cellulose molecule into glucose) [Haldar et al. 2018, Poszytek 2016]. As a result of their synergistic action they cause complete degradation of cellulose, while by acting selectively, in a controlled manner, they can lead to the production of specific products, e.g. low molecular weight products (such as glucose or cellobiose) or nanosized cellulose [Kafle et al. 2015]. The β-1,4-glycosidic bond in cellulose is broken as a result of a hydrolysis reaction involving cellulases such as endo-1,4-β-D-glucanase, exo-1,4-β-D-glucanase or β-1, 4-D-glucan-glucanhydrolase [Yeh et al. 2009, Żygo and Prochoń 2017].

Enzymatic hydrolysis as a method of nanocellulose production has many advantages, such as low cost of its implementation, no need to use harmful solvents, or the possibility of carrying out the process under mild conditions (temperature 45-50°C, pH = 4.8) [Żygo and Prochoń 2017]. It can also be used to treat biomass at the initial stage of its decomposition, where non-cellulosic products are initially removed from the raw material. For this purpose, the first step is to run a reaction with an enzyme complex that degrades lignin, hemicelluloses and pectin, followed by the next step involving enzymes that hydrolyze cellulose [Lee et al. 2014, Thapa et al. 2020]. The processes of enzymatic preparation of nanocellulose have great potential for commercialization, but unfortunately it is hindered by the high cost of recovering

enzymes for reuse [Druzhinina and Kubicek 2017, Sokołowska et al. 2016]. Enzymatic methods of nanocellulose preparation are relatively efficient and eco-friendly; however, further research is required to obtain nanocellulose in this manner, because the materials that are the products of such processes are not homogeneous, with nanometric particles constituting only a certain percentage of micrometric particles. Due to the fact that nanocellulose is increasingly commonly used in industry, very intensive work is being done on improving new technologies for its production [Michelin et al. 2020].

The aim of this study was to evaluate the effect of the duration of enzymatic hydrolysis on the properties of obtained nanocellulose, including its particle size, structure and crystallinity.

MATERIALS AND METHODS

Enzymatic hydrolysis

In this study cellulose was used in the form of Avicel PH-101 and Whatman cellulose filter paper No. 1 purchased from Sigma Aldrich Chemie (Darmstadt, Germany). The cellulose material (50 mg) was added to citrate buffer (1 ml, 50 mM, pH = 4.8) and pre-incubated while shaking for 30 min at 50°C using an incubated shaker (Lab Companion, JeioTech, Korea). The cellulolytic enzyme from the microscopic fungus *Trichoderma reesei* ATCC 26921 with an activity of 700 units/g (Sigma Aldrich Chemie, Darmstadt, Germany) was diluted in citrate buffer (1:50 by volume). Then, the enzyme in citrate buffer was mixed with cellulose materials (1: 2 by volume) and the obtained mixture was incubated at 50°C for 0.5, 1, 2, 4 hours with a shaking speed of 250 rpm/min. After that time the reaction was stopped by boiling the solution for 5 min. The samples were centrifuged (Universal 320, Andreas Hettich GmbH and Co. KG, Tuttlingen, Germany), washed with deionized water and dried in a laboratory dryer (Pol-Eko-Aparatura, Wodzisław Śląski, Poland).

As a result of the experiment using raw cellulose (Avicel and Whatman) eight different samples were obtained, the denotations of which are presented in Table 1.

Table 1. Symbols of cellulose samples

Type of cellulose	Time of enzymatic hydrolysis [h]	Designation of the sample
Avicel	0.5	A0.5
	1	A1
	2	A2
	4	A4
Whatman	0.5	W0.5
	1	W1
	2	W2
	4	W4

FTIR spectroscopy

Cellulose samples (1 mg) were mixed with KBr (200 mg) (Sigma Aldrich Chemie, Darmstadt, Germany) and analyzed in the pastille form using a Nicolet iS5 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

XRD analysis

The supermolecular structures of cellulose samples were analyzed using the X-ray diffraction (XRD) analysis (TUR M-62 X-ray diffractometer, Carl Zeiss AG, Jena, Germany) Deconvolution of peaks was performed using the method proposed by Hindeleh and Johnson

(1971) and improved and programmed by Rabiej (1991). The degrees of crystallinity for the cellulose samples were determined by comparing the areas under crystalline peaks and the amorphous curve.

DLS analysis

The particle sizes expressed as the hydrodynamic diameter of cellulose samples were determined using the DLS method (Zetasizer Nano ZS-90 instrument, Malvern, UK). Before analysis the tested materials were mixed with deionized water at a 2:5 ratio (w/v) and treated using an ultrasound bath (Polsonic, Warsaw, Poland) for 25 min.

RESULTS AND DISCUSSION

The FTIR spectra of Avicel and Whatman cellulose samples after enzymatic hydrolysis carried out at different time intervals are shown in Figs. 1 and 2.

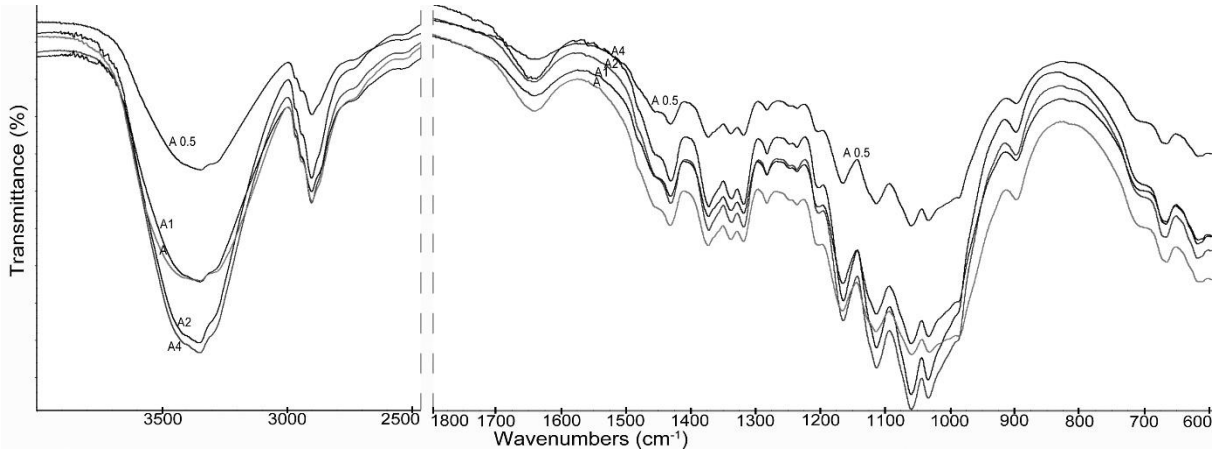

Figure 1. FTIR spectra of Avicel and Avicel cellulose treated with enzymes for 0.5, 1, 2 and 4 hours.

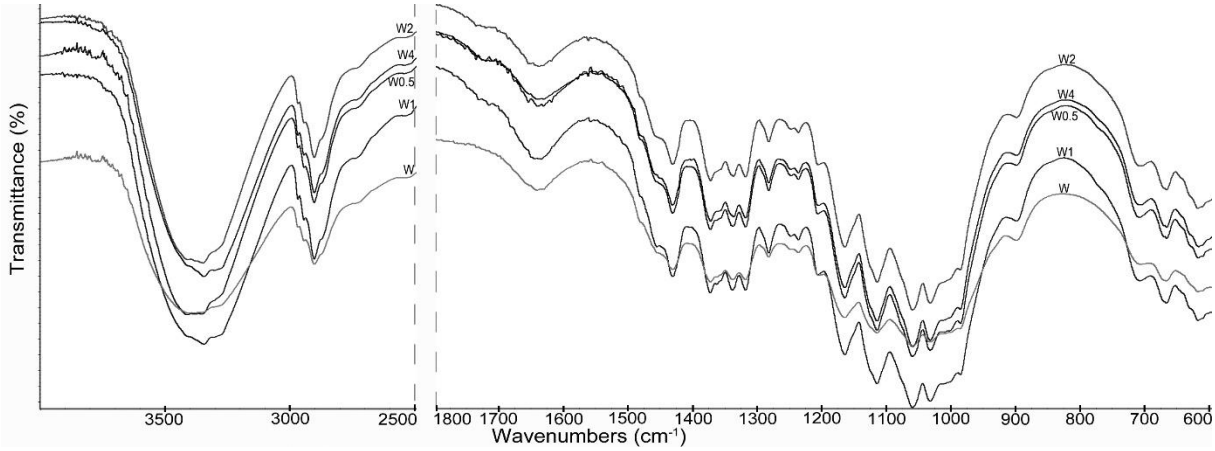

Figure 2. FTIR spectra of Whatman and Whatman cellulose treated with enzymes for 0.5, 1, 2 and 4 hours.

Typical peaks for cellulose are the bands at 3391 cm^{-1} (assigned to hydroxyl group stretching), 2906 cm^{-1} and 1373 cm^{-1} (assigned to stretching and deformation vibrations of the C-H group), 898 cm^{-1} (assigned to the β-glycosidic linkage) and 1061cm^{-1} (assigned to the -C-O- group) [Abderrahim et al. 2015]. All the above-mentioned bands are present also for cellulose samples after enzymatic hydrolysis. The range of 3200-3450 cm^{-1} corresponds to the stretching vibration of the -OH group. This band was observed for all the cellulose samples and

showed the lowest intensity for cellulose samples (Avicel and Whatman) after hydrolysis with enzymes in the process run for 0.5 h. The band at 2890 cm^{-1} is attributed to the C-H stretching vibration. The peaks at 1424 and 1363 cm^{-1} are attributed to asymmetric bending and wagging -CH_2. On the other hand, the 1152 cm^{-1} peak was assigned to the -C-O-C- bond (β-1,4-glycosidic bond) in cellulose [Pachuau et al. 2014]. Compared to raw cellulose it can be stated that the chemical structure of cellulose has been preserved. In a study by Zielińska et al. (2021), enzymatic hydrolysis of Sigmacell cellulose with the enzyme obtained from *T. reesei* run for 24 h caused only a reduction in the intensity of the bands for the obtained nanocellulose when compared to the raw material.

The diffraction profiles of cellulose samples subjected to enzymatic hydrolysis are shown in Fig 3.

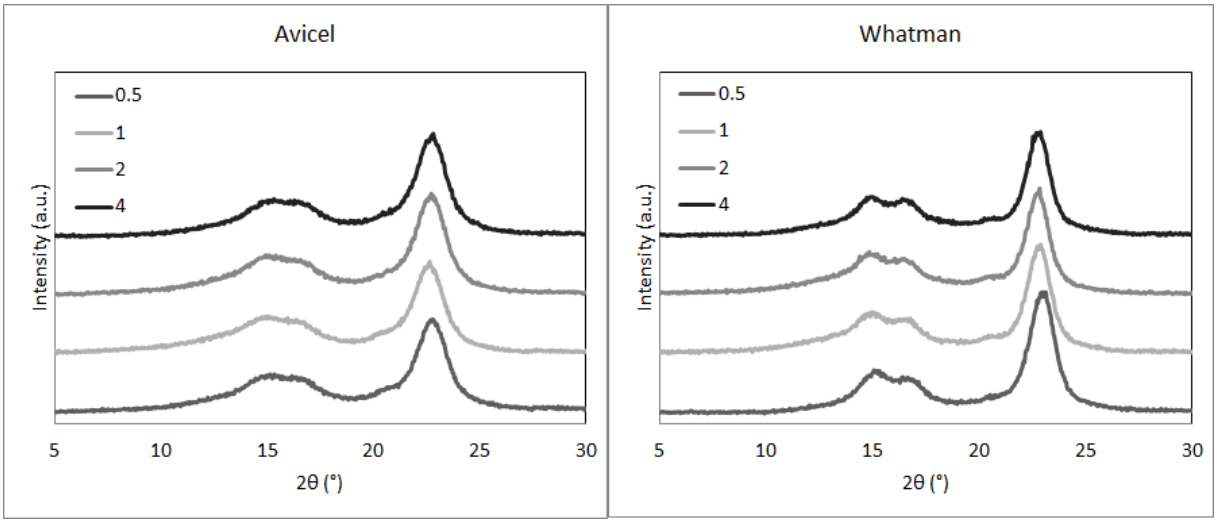

Figure 3. XRD patterns of Avicel and Whatman cellulose after enzymatic hydrolysis run for different times.

All the diffractograms of cellulose samples (in the form of Avicel and Whatman) after hydrolysis with enzymes showed three peaks at 2θ: 15° (1-10 plane), 17° (110 plane) and 22.7° (200 plane) [French 2014]. The presence of these peaks confirmed that the structure of cellulose I was preserved in the samples after enzymatic hydrolysis and this observation is consistent with the literature data [Babicka et al. 2021, Zielińska et al. 2021].

The calculations for the degree of crystallinity are presented in Table 2.

Table 2. The crystallinity index of cellulose samples

Samples	Crystallinity index [%]	Samples	Crystallinity index [%]
raw Avicel	65	raw Whatman	66
A0.5	67	W0.5	75
A1	67	W1	72
A2	69	W2	72
A4	69	W4	70

The crystallinity index of Avicel samples after hydrolysis with enzymes showed similar values (65-69%) regardless of how long the hydrolysis process lasted. Therefore, the enzymatic hydrolysis process did not significantly affect the percentage of the crystalline part in the Avicel cellulose samples. However, in the case of Whatman samples, an increase in the crystallinity

of the samples after the enzymatic hydrolysis process can be noticed, which is especially noticeable with short hydrolysis times.

In the next step of the study the particle size of the obtained nanocellulose was measured and the results are shown in Fig. 4.

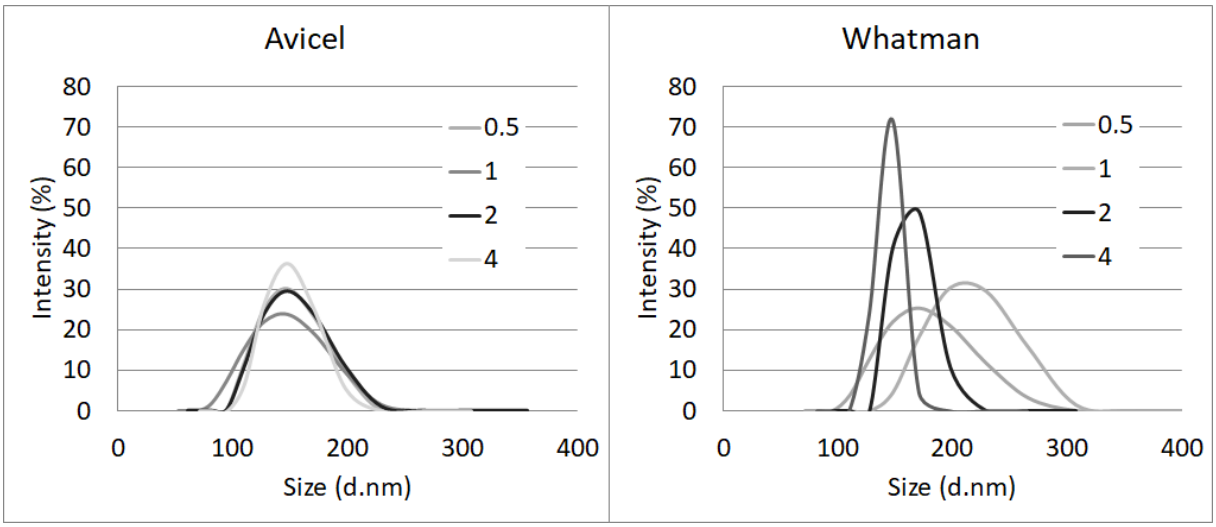

Figure 4. DLS patterns of Avicel and Whatman cellulose after enzymatic hydrolysis.

The enzymatic hydrolysis process of cellulose samples (Avicel and Whatman) significantly influenced their particle size when compared to the raw materials. The raw material was characterized by a particle size over 2000 nm and high polydispersity [Babicka et al. 2021]. In contrast, the Avicel cellulose after all the variants of enzymatic hydrolysis showed particle size of approx. 150 nm. In turn, Whatman cellulose after enzymatic hydrolysis run for 0.5 h was characterized by particles with an average dimension of approx. 230 nm. In the case of a longer hydrolysis process (t = 1, 2 and 4 h) the particle size of the obtained nanocellulose was about 150 nm. However, the particle sizes of the produced nanocellulose showed that the duration of the hydrolysis reaction had no significant effect on the mean particle diameter.

CONCLUSIONS

In this study the effect of the duration of enzymatic hydrolysis on the properties of the obtained nanocellulose, including its particle size, structure and crystallinity, was evaluated. The obtained nanocellulose, regardless of the time of enzymatic hydrolysis, was characterized by a similar particle size (except for the Whatman cellulose sample subjected to hydrolysis for 0.5 h) as well as a similar structure and degree of crystallinity. The results presented in the paper showed that the enzymatic hydrolysis time did not affect the tested parameters of the obtained nanocellulose. Moreover, the use of cellulolytic enzymes produced by *Trichoderma reesei* fungi is a promising method to obtain cellulose with nanometric dimensions.

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Streszczenie: *Wpływ czasu procesu hydrolizy enzymatycznej na właściwości nanocelulozy*. Celem pracy było określenie wpływu czasu hydrolizy enzymatycznej na właściwości otrzymanej nanocelulozy. W badaniach wykorzystano dwa materiały celulozowe do produkcji nanocelulozy: Avicel i Whatman. Do przeprowadzenia reakcji hydrolizy enzymatycznej zastosowano enzym celulolityczny uzyskany z grzyba *Trichoderma reesei*. Hydrolizę enzymatyczną przeprowadzono na celulozie stosując czas reakcji wynoszący 0,5, 1, 2 i 4 godziny. W celu scharakteryzowania otrzymanych materiałów zastosowano następujące analizy: spektroskopię w podczerwieni, dyfrakcję rentgenowską oraz dynamiczne rozpraszanie światła. Uzyskane wyniki wykazały, że celuloza po hydrolizie enzymatycznej wykazywała podobne parametry (wielkość cząstek, strukturę nadcząsteczkową i stopień krystaliczności) po wszystkich zastosowanych czasach reakcji.

Słowa kluczowe: nanoceluloza, hydroliza enzymatyczna, XRD, FTIR, DLS

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