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Research into Isolation of Cellulose Micro- and Nanofibres from Hemp Straw Using Cellulolytic Complex from *Aspergillus niger*

Abstract
Cellulose micro- and nanofibers were produced on a base of pulp obtained as a result of thermal-mechanical and chemical pretreatment of hemp straw. Isolation of micro-and nanofibres was carried out using the enzymatic treatment with the use of a cellulolytic complex of Aspergillus niger and two-stage enzymatic and mechanical treatment. Nanofibres obtained by intense enzymatic treatment were characterized by a diameter of less than 100 nm. As a result of the two-stage enzymatic-mechanical treatment micro-and nanofibres were obtained with a diameter in the range of 25-400 nm and a length of several hundred nanometers to several micrometers.

Key words: *cellulose nanofibres, hemp straw, cellulolytic complex, mechanical disintegration.*

Introduction

Cellulose is the main biopolymer derived from biomass. It is estimated that the annual production of cellulose in the biosphere falls in the range of tens to a hundred billion tons [1]. Of this huge amount only about 6 billion tons of cellulose is processed by various industries such as paper, textile and chemical industries [2]. Although cellulose is the main structural constituent of wood it also occurs in other plant fibres, such as cotton, flax or hemp, in marine organisms – tunicates, in algae and fungi or it is produced by some bacteria. On the other hand, plant derived cellulose may occur in various parts of plants such as the trunks of trees or plant stems (flax, hemp) in the leaves (sisal) or in fruits (cotton).

Cellulose is an important component of the cell wall as it forms a backbone, on which the other cell wall components such as hemicelluloses, lignins and pectins are deposited during the growth of the cells [3]. Cellulose macromolecules are synthesized by the cell membrane. They are spatially ordered thanks to the intra- and intermolecular hydrogen bonds. Each cellulose chain is stabilized by intermolecular hydrogen bonds formed between the oxygen atom in the pyranose ring of one anhydroglucose residue and the hydrogen atom in the hydroxyl group at the C3 carbon atom in the next anhydroglucose residue, and between the hydroxyl groups on C2 and C6 carbon atoms of the adjacent anhydroglucose residues [4]. Cellulose is characterized by a hierarchical structure reflecting its biological origin. Thirty-six individual cellulose chains are combined with each other to form the so-called microfibrils or elementary fibrils with a diameter of 3.5 nm, which, in turn, are arranged in bundles of 20 - 50 nm in diameter, forming a structure known as microfibrillar cellulose. These bundles are the component parts of the cellulose fibres [5, 6].

Recent years have seen a tremendous growth of research on the preparation of composite materials using nano-scale fillers (below 100 nm). Among the different types of nanomaterials used to reinforce polymer matrices cellulose nanocrystals and nanofibres deserve special attention.

There are different methods of microfibrils isolation from cellulose fibres [7]. In one method a suspension of cellulose fibres is treated in a high pressure homogenizer. Pressure from a few hundred to as much as 2000 bar is dramatically reduced in the controlled gap homogenization valve, causing a turbulent flow which breaks the fibres into fibrils under the influence of giant shear stress. However, this method is energy-intensive, and is primarily used for the production of small amounts of nanofibres [8]. Another method is the hydrolysis of cellulose with concentrated sulfuric or hydrochloric acids which brings about the break-

Table 1. *Activity characteristic of the enzyme complex from A. niger.*

Enzyme activity	Value, U/cm3
Endo-1,4-ß-glucanase	511.60
FPA	11.60
ß-glucosidase	409.40
Exo-cellobiohydrolase	20.44
Endo-1,4-ß-xylanase	1008.50
Polymethylgalacturonase	312.70
Pectin lyase	2.93
Pectinesterase	96.02

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down of an amorphous cellulose, leaving the so-called nanowhiskers or cellulose nanocrystals. There have been reports on the effect of treatment of cellulose pulp by hydrolytic enzymes like cellulases and xylanases on its dissolution ability in an aqueous sodium hydroxide [9 - 11]. The aim of the study undertaken by the authors was to assess the potential use of enzymatic treatment as a direct method for obtaining micro-and/or nanofibres of cellulose derived from hemp straw or as a pretreatment to facilitate the desintegration of fibres by mechanical treatment.

Materials and methods

Materials

In this work the straw of hemp (*Cannabis sativa L.*) was used which had been cultivated at Agricultural University of Cracow. The straw was subjected to thermomechanical and chemical pretreatment at Institute of Biopolymers and Chemical Fibres. The straw pretreatment consisted in steam treatment in an autoclave, grinding in a Sprout-Waldron mill and then digestion in a solution containing 5.0% NaOH and 5.5% hydrogen peroxide in relation to an absolutely dry pulp.

Pulp extracted from the hemp straw (symbol SK12) was characterized by the following parameters: average viscosimetric degree of polymerization (DP) of 855, α-cellulose content of 82.3%, lignin content (Kappa number) of 58.3. Enzymatic treatment of pulp was carried out using multi-enzyme cellulolytic complex from *Aspergillus niger,* developed at the Institute of Biochemistry, Lodz University of Technology. The characteristic of enzymatic activities is shown in ***Table 1***.

Methods of obtaining micro-and nanofibres

Enzymatic treatment

Conditions of enzymatic treatment were chosen according to the authors' previous experience. Cellulolytic complex in an amount of 50 - 1000 U of β-glucanase activity per 1 g of the dry weight of cellulose was added to 1.5 l of a 5 wt% suspension of cellulose pulp in 0.05 M acetate buffer of pH 4.8 and the whole was incubated at 50 °C for 6 hours in the Labfors incubator (Infors, Switzerland) with shaking at a frequency of 150 cycles/min. Then the pulp was filtered from the reaction liquid and the residue of the enzyme was inactivated by autoclaving at 121 °C for 20 minutes.

Table 2. *Average molecular weight and average polymerization degree of cellulose before and after enzymatic treatment by A. niger cellulolytic complex.*

Variant/ Sample	Enzyme/ substrate ratio, U/g	Average Mn, kD	Average Mw, kD	Mw/Mn, –	Average DP, –
SK12	–	22.4	138.6	6.2	855
1/SK12-1000	1000	17.0	58.1	3.4	359
2/SK12-50	50	19.5	105.0	5.4	648
3/SK12-200	200	18.3	85.6	4.7	513

Table 3. *Selected properties of cellulose pulp before and after enzymatic treatment by A. niger cellulolytic complex.*

Variant/ Sample	Enzyme/ substrate ratio, U/g	Reducing sugars, mg/g	Glucose, mg/g	Weight loss, %	Alpha-cellulose, %	Kappa number, –
SK12	–	–	–	–	82.3	58.3
1/SK12-1000	1000	285.8	154.6	32.4	87.1	88.8
2/SK12-50	50	94.8	20.3	9.0	83.6	64.1
3/SK12-200	200	176.6	51.2	14.8	84.8	62.0

Mechanical disintegration

After the enzymatic treatment 500 cm^3 of 2 wt% cellulose fibre suspension in deionized water was subjected to mechanical disintegration with the use of T50 homogenizer equipped with the S50N-G45G dispersing tool (IKA, Germany) at a rotational speed of 10 000 r.p.m. for 10 min.

Analytical methods

Reducing sugars determination

The content of reducing sugars in the post-reaction solution was determined by a colorimetric method with 3,5-dinitrosalicylic acid using calibration curve prepared for D-glucose [12].

Glucose content determination

Glucose content in the post-reaction solutions was determined by a colorimetric metod with glucose oxidase (GOD) using calibration curve prepared for D-glucose [13].

Alpha-cellulose content determination

Biomass sample in an amount of 3g was soaked for 30 min in 15 cm^3 of 17.5% sodium hydroxide solution, washed with distilled water and assayed by weighing according to PN-P-50099:1962 standard [14].

Kappa number determination

Kappa number which is a measure of the lignin content of the pulp was determined by titration in accordance with TAPPI T 236 cm-85 standard [15].

Analysis of the morphological structure and dimensions of fibres

The morphology of obtained cellulose micro-and nano-fibres was analyzed by Quanta 200 scanning electron microscope (FEI, USA) at an accelerating voltage of 30 kV. Before the SEM analysis the dried samples were gold-sputtered by Q150RS high vacuum sputter coater (Quorum Technologies, UK). For the analysis of the diameter and length of the micro-and nanofibres in digital images, MultiScan computer image analysis system (Computer Scanning Systems, Poland) was used.

Cellulose polymerization degree determination

The polymerization degree of cellulose was determined by viscosimetric method, after dissolving the sample in a solution of the iron-tartaric complex (EWNN) according to PN-P-50101-02:1992 standard [16].

Cellulose molecular weight distribution

Determination of the molecular weight distribution and average molecular weight of cellulose micro- and nanofibres was carried out by gel permeation chromatography (GPC/SEC) using the HP 1047 differential refractive index detector (Hewlett-Packard, USA). Samples of 20 mg were prepared according to a procedure based on the Ekmanis method [17].

Results and discussion

Molecular weight distribution curve of initial pulp SK12 is bimodal (***Figure 1,*** see page 42), with two dominant fractions, having the maxima for the molecular weight of about 10,000 and about 180,000. This is also the reason for the high value of the Mw/Mn polydispersity index (***Table 2***).

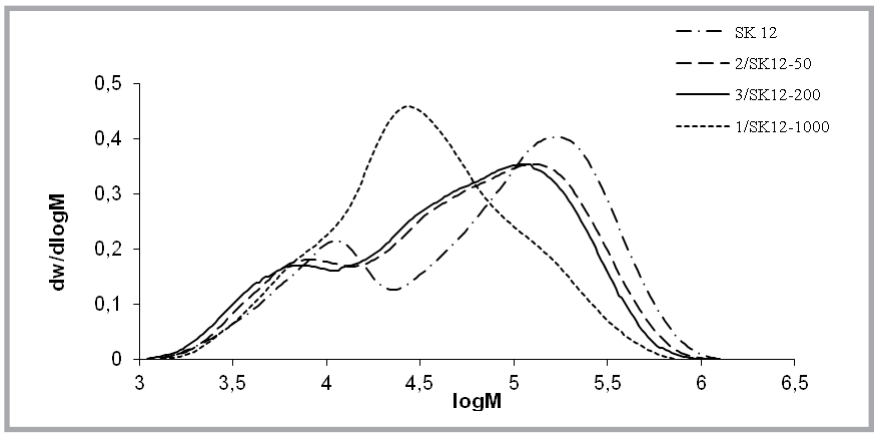

Figure 1. *Molecular weight distribution curves of pulp from straw hemp: before treatment (SK12), after 1000 U/g enzymatic treatment (1/SK12-1000), after 50 U/g enzymatic treatment and mechanical disintegration (2/SK12-50) and after 200 U/g enzymatic treatment and mechanical disintegration (3/SK12-200).*

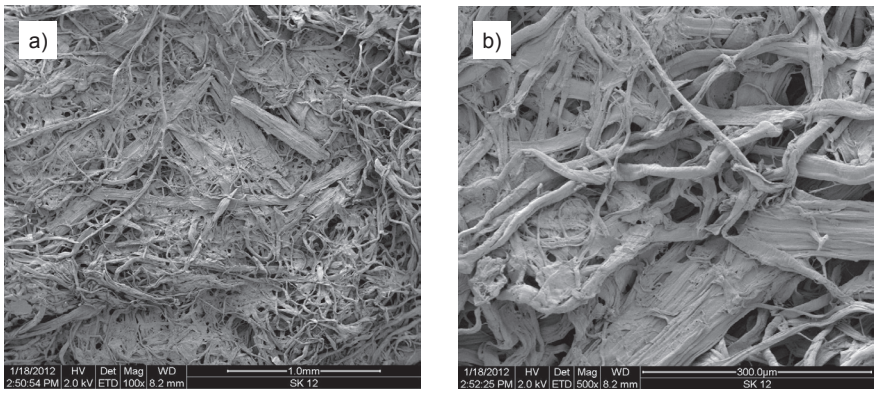

Figure 2. *SEM photographs of initial cellulose pulp SK12 extracted from hemp straw.*

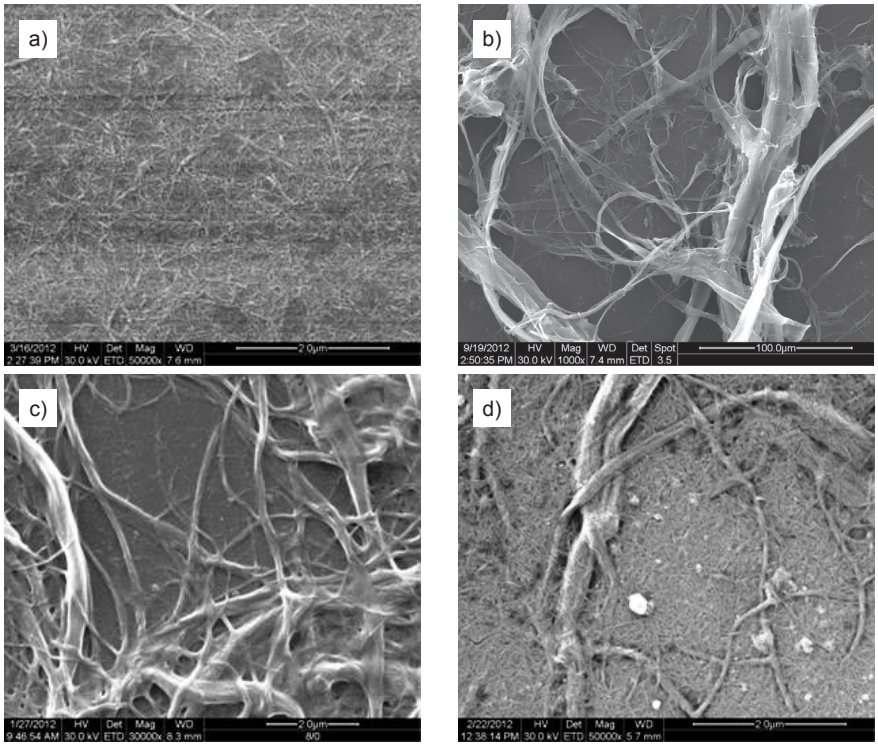

Figure 3. *SEM photographs of cellulose micro- and nanofibres resulting from: a) enzymatic treatment 1000U/g (1/SK12-1000), b) mechanical disintegration (SK12/M), c) enzymatic treatment 50U/g and mechanical disintegration (2/SK12-50), d) enzymatic treatment 200U/g and mechanical disintegration (3/SK12-200).*

The alpha-cellulose content amounting to 82.3% and a relatively high value of the Kappa number indicates that the pulp still contains portion of hemicelluloses and lignin (***Table 3***, see page 41).

The cellulose fibres in the initial pulp were characterized by diameters in the range 5-150 micrometers and a length of several micrometers to several millimeters (***Figure 2***).

Three variants of treatment were used in the study in order to obtain cellulose nanofibres.

In the first variant an attempt was made to obtain cellulose nanofibres with a single-stage enzymatic treatment using enzyme/substrate ratio equal to 1000 units of endo-1,4-β-glucanase activity per 1 g of absolute dry pulp (***Table 2***). There was a significant decrease in the average degree of polymerization of 58% and a reduction of number and weight average molecular weight of cellulose (***Table 3***). The shape of molecular weight distribution curve changed from a bimodal to that resembling the Gaussian normal distribution, characteristic peak for the fraction having a molecular weight of about 10,000 faded, and the entire curve shifted toward the lower molecular weight (***Figure 1***). The alpha-cellulose content in enzyme treated pulp increased in relation to the initial pulp of about 5%. This may indicate that the enzymatic treatment resulted in a hydrolysis of hemicellulose component. Lignin component in cellulose is not hydrolyzed by the enzyme complex, and therefore relative contribution of lignin in the pulp increases. A disadvantage of this option is a significant weight loss of pulp which amounts up to nearly 1/3 of its initial weight (***Table 2***). A high glucose content in reducing sugars released in the enzymatic reaction (***Table 2***) is a consequence of the relatively high activity of β-glucosidase in the enzyme complex (***Table 1***).

On the SEM photograph (***Figure 3.a***) it can be seen that as a result of intensive enzymatic treatment of the pulp cellulose nanofibres were isolated having a diameter in the range of 25 - 50 nm and a length/diameter ratio in the range of 10 - 45.

In the second variant an enzymatic treatment using enzyme/substrate ratio of 50 U/g was applied, followed by a mechanical disintegration using a ho-

mogenizer. In this case, an average degree of polymerization in the pulp was reduced by about 24%. The molecular weight distribution curve retained bimodal character, but it has been shifted in the direction of lower values. There was an increase in alpha-cellulose content and in the Kappa number (***Table 3***) however, much lower than in the case of the SK12/1000 experiment. SEM image analysis showed that, as a result of the applied two-step enzyme-mechanical treatment, it was able to obtain cellulose nanofibres having a diameter of 60 - 100 nm and a length/diameter ratio of 50 - 70 (***Figure 3.c***). However, in a microscopic image, also some objects exist with a diameter of 100 to 300 nm and a length up to 8 microns, which may be the result of incomplete fibrillation of cellulose fibres. For comparison, ***Figure 3.b*** shows the SEM image of the SK12/M pulp subjected only to mechanical disintegration. As is apparent from the analysis of the image, in this case only microfibres having a diameter in the range 0.3 - 12 microns and 25 - 250 microns in length were obtained.

In the third variant the pulp was also subjected to the two-step treatment as in the second variant, except that a higher enzyme/substrate ratio of 200 U/g was used. The average degree of polymerization of the pulp after that treatment was reduced by 40% compared to the initial value. The molecular weight distribution curve had a similar pattern as for the second variant, however, it was slightly shifted towards lower values. The content of alpha-cellulose, and the value of Kappa number also increased compared to the initial pulp and they were at the levels similar to the second variant (***Table 3***). The analysis of the SEM images shows that in the result of the applied enzymatic-mechanical treatment, cellulose nanofibres with diameters in the range of 25 - 100 nm and a length/diameter ratio of 15 - 45 were obtained (***Figure 3.d***). In the image also few fibrils with diameters in the range of 100-400 nm and lengths of several micrometers were observed.

Conclusions

As a result of enzymatic treatment (1000 U/g) of pulp derived from hemp straw it was possible to obtain cellulose nanofibres having a diameter less than 100 nm and a length/diameter ratio in the range 10 - 45. Two-step enzymatic-mechanical treatment has led to nanofibres with diameters in the range 60 - 100 nm and a length/diameter ratio in the range 50 - 70 for 2/SK12-50 variant using the enzyme preparation in an amount of 50 U/g and with a diameter of 25 - 100 nm and a length to diameter ratio in the range 15 - 45 for 3/SK12-200 variant using the enzyme preparation in an amount of 200 U/g.

The use of a single-step mechanical disintegration did not lead to nanofibres but only resulted in partial fibrillization of cellulose fibres. This is in an agreement with the reports [10, 11], that the action of the cellulolytic complex on cellulose causes not only a reduction in its molecular weight by random cleavage of β-glucosidic bonds along the cellulose macromolecules or by splitting glucose or cellobiose residues at the ends of chains but also contributes to the reduction of the number and/or energy of intermolecular hydrogen bonds. That fact probably facilitates the penetration of water molecules into cellulose and better swelling of the cellulose fibres and thereby facilitates their further, mechanical disintegration. Dimensions of isolated nanofibres indicate the possibility of their use as a filler in the manufacture of cellulosic composite materials. As a result of a biorefining of cellulose with the use of enzyme from *A. niger* cellulose nanofibres are produced along with some soluble products which can be used as a feedstock for the production of renewable energy sources such as ethanol.

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