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Study of enzymatic hydrolysis of bacterial nanocellulose

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Abstract : Bacterial nanocellulose is a subject of extensive research due to its promising potential applications in medicine, veterinary and cosmetics. In this paper, effect of structural characteristics on enzymatic hydrolysis of bacterial nanocellulose (BNC) and microcrystalline cellulose (MCC) has been studied. Despite the similar degree of crystallinity and lateral size of crystallites for both cellulose samples, a conversion degree of BNC after enzymatic hydrolysis was considerably higher than of MCC. The main distinctive feature of the BNC sample is a high porosity and developed surface of nanofibrils. As against, the MCC sample contains coarse low-porous particles that have a poorly developed surface. Although drying reduces the porosity of the samples, the dry BNC retains a much higher pore volume and greater enzymatic hydrolysability than the dry MCC. Due to highly porosity and developed surface, the BNC sample acquires a high accessibility to molecules of cellulolytic enzymes that promotes enzymatic hydrolysis of this sample both in never-dried and dry state.

Keywords : Bacterial nanocellulose; Crystallinity; Porosity; Specific surface; Enzymatic hydrolysis.

INTRODUCTION

Among various organic materials, cellulose is a most appropriate for preparation of various types of nanomaterials, since this abundant natural polymer has a nanostructured organization and specific properties such as low density, hardness and abrasivity; ability to structural and chemical modification; biocompatibility; biodegradability in the nature, etc. Currently, several kinds of nano-scale cellulose materials are known such as nanofibers, nanoparticles and bacterial nanocellulose[1-4].

Despite of abundant investigations, some features of the nanocelluloses have been not studied yet, and namely a transformation process of cellulose into glucose under effect of cellulolytic enzymes, and dependence of enzymatic cleavage on structural parameters of the nanocellulose. As is known, any kind of the nanocellulose has a developed specific surface that may contribute to enzymatic hydrolysis. However on the other hand, a high crystallinity of the nanocellulose is an important factor that might hinder the enzymatic cleavage. To solve the problem, what structural factor plays a prevalent role in enzymatic decomposition, various nanocelluloses can be investigated. However, such kinds as nanofibers, nanofibrillated celluloses and nanoparticles are less suitable for this purpose due to low purity, presence of extraneous

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admixtures (oligosaccharides, hemicelluloses, lignin, extractives, salts, etc.) and non-cellulosic functional groups (carbonyl, carboxyl, sulfonic, etc.) that can distort the experimental results. The most suitable model to solve this problem is bacterial nanocellulose (BNC), which in contrast to other kinds of nanocellulose does not contain non-cellulosic admixtures and functional groups.

The BNC is a subject of continuing research and has great commercial interest in terms of manufacturing of new biomedical products having improved biocompatibility and biodegradability, etc. Main application areas of the BNC can be cosmetics (e.g. moistening masks and creams), as well as medicine and veterinary (e.g. nano-implantants)[1,5].

The BNC is produced by several species of ubiquitous fermentation bacteria, most importantly *Gluconacetobacter xylinus*[5,6]. The forming long and thin elementary nanofibrils having lateral size of 7-10 nm are aggregated to microfibrils. The neighboring microfibrils interlace and form bands having spongy structure filled with water; therefore, the never-dried BNC contains extremely high content of the embedded water, up to 99%. The formation of BNC by fermentation opens up new vistas for the in situ shaping of cellulose. This bio-shaping allows obtaining pellicles, beads, fibres and hollow bodies by changing the conditions of the bacteria cultivation. The bacterial nanocellulose is characterized by high purity, developed surface area, enhanced crystallinity, high content of CI_{α} crystalline allomorph and increased degree of polymerization[1,6,7]. To discover the effect of nanostructure on the enzymatic digestibility, it is advisable to compare the nanocellulose with another cellulose sample having no nanostructure.

Main purpose of this paper was to study the degree transformation of bacterial nanocellulose into glucose under effect of cellulolytic enzymes compared with microcrystalline cellulose.

EXPERIMENTAL

Materials

Never-dried sample of BCN was kindly provided from Department of Chemistry and Earth Sciences of Institute of Organic and Macromolecular Chemistry, Friedrich Schiller University, Jena, Germany. To remove surplus of water, the never-dried BNC sample was centrifuged at 3000 *g* for 15 min.

Besides, the sample of microcrystalline (MCC) cotton cellulose was investigated. The never-dried MCC was prepared by hydrolysis of cotton cellulose with boiling 2.5N HCl for 1 h; the hydrolyzed cellulose was neutralized and washed to pH=7. To remove surplus of water, the never-dried MCC sample was centrifuged at 3000 *g* for 15 min.

To obtain of dry celluloses, the never-dried samples were centrifuged to remove surplus of water, rinsed with absolute ethanol and dried at 60°C for overnight.

Enzymatic hydrolysis

The cellulose samples were enzymatically hydrolyzed to determine their accessibility to cellulolytic enzymes. For this purpose we used the commercial cellulase preparation Cellic Ctec-2 produced by Novozymes A/S, Bagsvaerd, Denmark. Enzymatic hydrolysis was carried out in 20-mL glass bottles each containing samples with loading of 10 g/L in 50 mM acetate buffer (pH=4.8). The samples were thoroughly mixed with the buffer and then Ctec-2 was added to obtain dose of 10 mg enzyme per 1g of dry cellulose. The closed bottles were placed in a shaker incubator at 50°C and agitated at 150 rpm during 10-72 h.

The concentration of the glucose (C_g, mM) obtained as a result of enzymatic hydrolysis of the cellulose samples was determined by HPLC-apparatus of Agilent Technologies 1200 Infinity Series using the Amines HPX-87H column. Main conditions of the HPLC-analysis were: temperature 45°C; mobile phase 0.005 M sulfuric acid; flow rate 0.6 ml/min. The hydrolyzate was preliminary filtered through 0.45 μm Nylon filter and degassed. Conversion degree (CD) of cellulose samples at enzymatic hydrolysis was calculated by the equation[8]:

$$CD = 100\% (C_g/C_{g,m}) \quad (1)$$

where $C_{g,m}$ is the maximum concentration of glucose at 100% conversion of cellulose.

Wide-angle X-ray scattering (WAXS)

WAXS- diffractograms of the samples were recorded in the 2Θ angle range from 5 to 50° using a diffractometer "Rigaku-Ultima Plus" (CuK_{α} – radia-

tion, λ=0.15418 nm). After recording of the diffractograms, the background was separated, and selected X-ray patterns were corrected and normalized. Then diffraction intensities from crystalline and non-crystalline regions were separated by a computerized method. The degree of crystallinity (X) was calculated according to equation[7,9]:

$$X = \int I_{cr}\, d\theta / \int I_o\, d\theta = F_{cr}/(F_{cr} + F_{am}) \quad (2)$$

where I_o is total intensity of the corrected diffractogram after subtraction of the parasitic background; I_{cr} is intensity of the crystalline scattering; F_{cr} is area of the crystalline scattering; F_{am} is area of the amorphous scattering.

The lateral size of crystallites (L) was determined by an improved WAXS-method[7,10]. The reflection at $2\Theta_o$ 22.5-22.7° was isolated, its integral width (B) in radians was measured, and corrections for instrumental factor (Δ) and lattice's distortion (δ_d) were introduced. The L-value was calculated by the equation:

$$L = \lambda/[(\cos\Theta_o (B^2 - \Delta^2)^{0.5})^2 - (2\delta_d \sin\Theta_o)^2]^{0.5} \quad (3)$$

Optical microscopy (OP)

OP images were obtained using a universal computerized optical microscope "Eclipse LV-UDM". The never-dried sample was freeze-dried and ground in a "Waring"-mill at 10000 rpm for 1 min. The dry MCC powder was placed onto an object glass and investigated.

Scanning electron microscopy (SEM)

SEM images were obtained using an electron microscope "Hitachi S-4700". The never-dried sample was preliminary freeze-dried, and then its micro-section was coated with monomolecular layer of gold. The micro-section was placed in a microscope, evacuated and electronic image was obtained.

Porosity

Porosity of the samples or total volume of pores (V_p) in the water medium was tested by method of water retention value (WRV) using a centrifugal force 3000 *g* for 15 min[11,12].

RESULTS AND DISCUSSION

Kinetic curves of enzymatic hydrolysis of cellulose samples are shown on Figure 1 and 2. These curves are characterized by a fast increase in the conversion degree (CD) of cellulose into glucose during 20-24 h and followed by reducing in the rate of hydrolysis. As follows from the investigations, the final conversion degree of never-dried BNC after enzymatic hydrolysis was about 1.6 times higher than of never-dried MCC. After drying, the enzymatic digestibility of both cellulose samples is reduced, but the final conversion degree of the dry BNC remains about 1.5 times higher than of the dry MCC sample.

To explain the results of enzymatic hydrolysis it is expedient to study the structural characteristics of the cellulose samples. SEM investigations show that the BNC has a network structure consisting of interwoven long and thin nano-fibers with average diameter of about 10 nm (Figure 3). In contrast to bacterial nanocellulose,

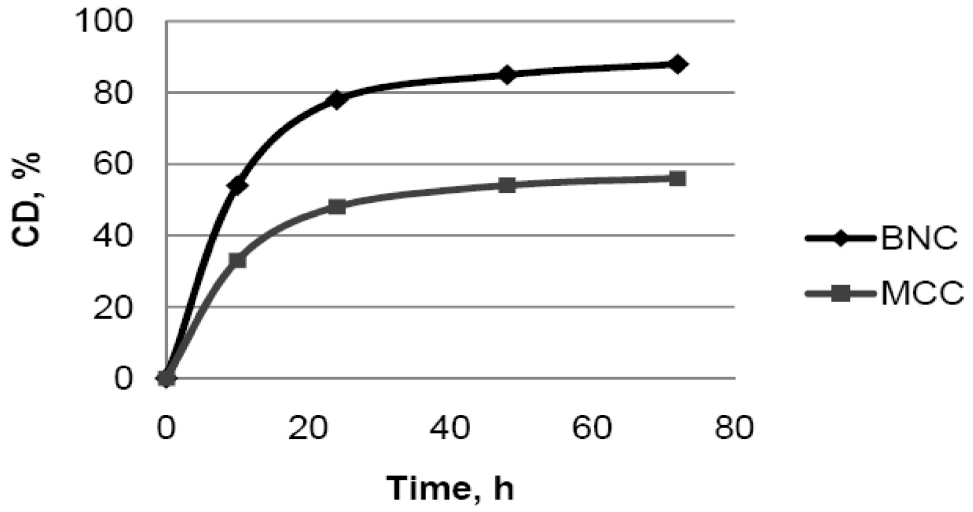

Figure 1 : Kinetic curves of enzymatic hydrolysis of never-dried cellulose samples

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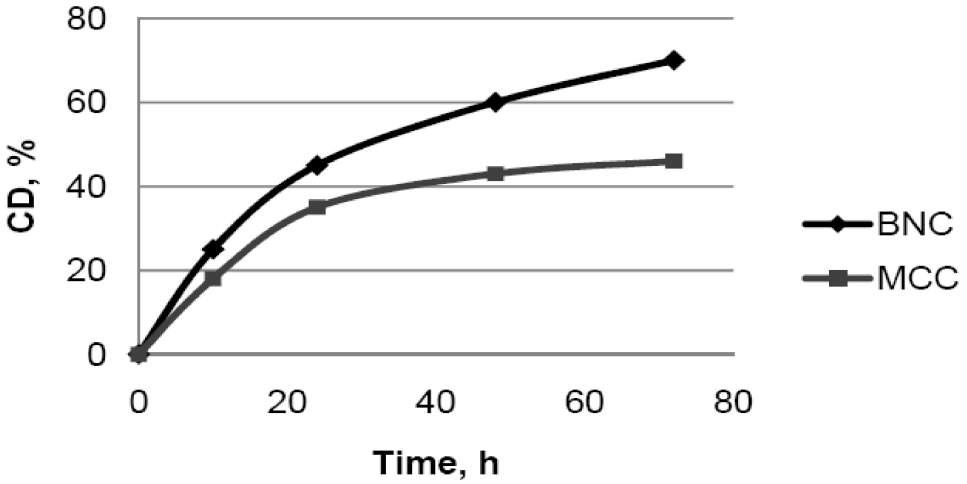

Figure 2 : Kinetic curves of enzymatic hydrolysis of dried cellulose samples

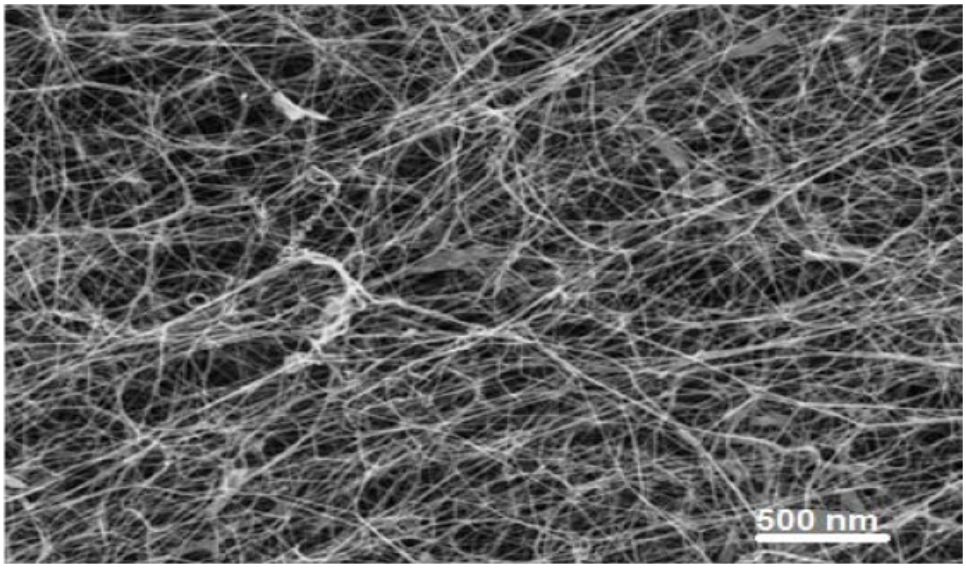

Figure 3 : SEM-image of bacterial nanocellulose

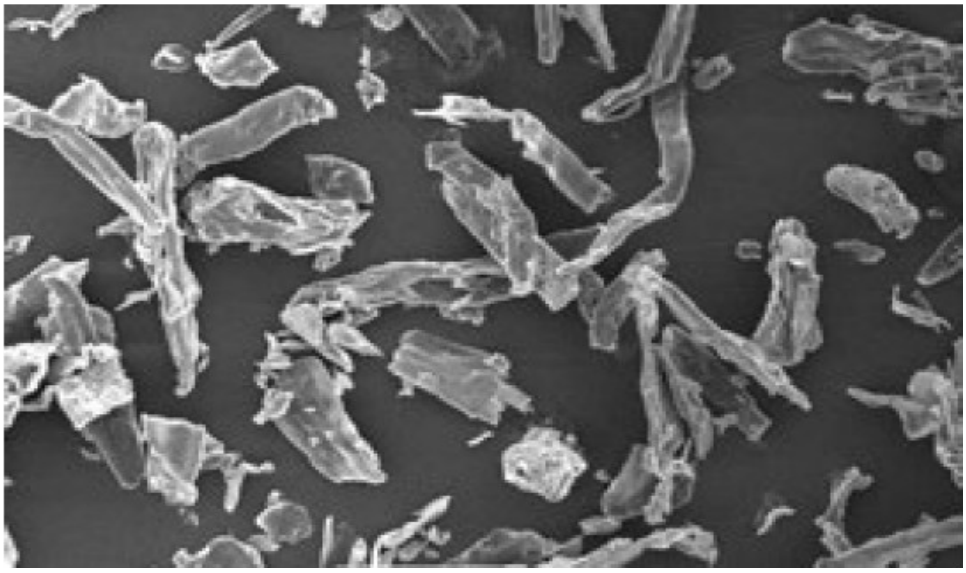

Figure 4 : Micro-photograph of microcrystalline cellulose (magnification 300 x)

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the microcrystalline cellulose contains coarse particles with a length of 50-100 μm and width of 20-30 μm (Figure 4).

On the basis of average diameter (D) of the BNC nanofibers and MCC particles, the specific external surface of the samples can be calculated:

$$S_{sp} = 6k/D \quad (4)$$

where k is coefficient of dimensionality.

As can see from TABLE 1, the BNC has a well-developed specific surface. The developed surface area of bacterial nanocellulose is correlated with high porosity of this sample. Moreover, these structural parameters of BNC greatly exceed characteristics of MCC.

As is known, the crystallinity is an important factor affecting the enzymatic cleavage of cellulose[7,8]. However, from structural investigations follows that both BNC and MCC samples have similar crystallinity degree (0.76-0.78) and lateral size of crystallites (8-9 nm). Despite this fact, the enzymatic cleavage of BNC was much higher than of MCC (Figure 1, 2).

The main distinctive feature of BNC is that it consists of a highly porous network of nanofibrils having a developed surface (TABLE 1, Figure 3). As against, the MCC sample contains low-porous micron-scale particles that have a poorly developed surface (TABLE 1, Figure 4). Although drying reduces the porosity of the both samples, the dry BNC retains a much higher pore volume and greater enzymatic hydrolysability than the dry MCC (Figure 2, 5).

Thus, highly accessible nano-dispersed structure is a main factor promoting enzymatic conversion of bacterial nanocellulose into glucose both in never-dried and dry state.

TABLE 1 : Structural characteristics of cellulose samples

Characteristics	BNC	MCC
Crystallinity	0.78	0.76
Lateral size of crystallites, nm	8	9
Specific surface, m^2/m^3	$6x10^8$	$2x10^5$
Porosity, cm^3/g	23 (30)*	0.8 (2.2)*

***Note: The porosity for never-dried samples was shown in brackets**

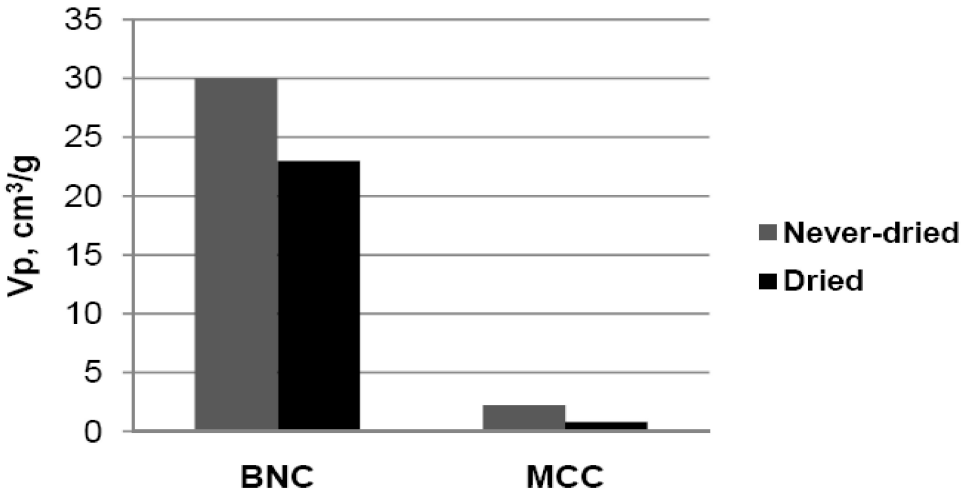

Figure 5 : Porosity of the never-dried and dry cellulose samples

CONCLUSION

Effect of structural characteristics on enzymatic hydrolysis of bacterial nanocellulose and microcrystalline cellulose has been studied in this paper. Despite the similar crystallinity degree and lateral size of crystallites for both cellulose samples, the conversion degree of BNC after enzymatic hydrolysis was considerably higher than of MCC. This fact can be explained by the well-developed porosity and surface of the nano-structured bacterial cellulose. Due to these structural features, the BNC sample acquires a high accessibility to molecules of cellulolytic enzymes that promotes enzymatic hydrolysis of this sample both in never-dried and dry state.

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