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Supramolecular Structure - A Key Parameter for Cellulose Biodegradation

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Summary: Three different cellulosic substrata, like microcrystalline cellulose, cotton cellulose and spruce dissolving pulp, were chosen for biodegradation. The kinetics of the enzymatic hydrolysis of these celluloses by *Trichoderma reesei,* has been investigated. The experiments proved the fact that both the morphological structure and the crystalline one are crucial to the process and the ratio of the reactions. In addition, in order to obtain the most accessible cellulose substratum it was studied the biodegradation of cellulose allomorphs of spruce dissolving pulp. The insoluble cellulose fraction remaining after enzymatic hydrolysis was examined by X-ray diffraction method and it was established the degree of crystallinity and the average crystallite size. The enzymatic degradation is also proved by the decrease in the degree of polymerization of hydrolyzed samples.

Keywords: biodegradation; cellulose; degree of polymerization (DP); enzymes; wide-angle X-ray scattering (WAXS)

Introduction

Cellulose, the most abundant component of plant biomass, is found in nature almost exclusively in plant cell walls, although it is produced by some animals (e.g., tunicates) and a few bacteria.[1,2] Cellulose is synthesized in nature as individual molecules (linear chains of glucosyl residues) which undergo self-assembly at the site of biosynthesis.[3] The enzymatic degradation of cellulose [β(1–4)-linked glucose] is of large biological and economical importance. Cellulose biodegradation studies emphasized the importance of improved cellulosic technologies and better cellulolytic enzymes for conversion of biomass to easily fermentable compounds such as glucose.[4–8] Cellulose biodegradation studies have been generally oriented upon physiological characteristics of cellulolytic microorganisms and also on biochemical properties of the enzymes synthesized by them.[9–11] Moreover, the enzymatic chain cleavage has received attention by significant contribution to elucidation of cellulose structure, as well as characterization of cellulose derivatives.[12] Enzymatic degradation of crystalline polysaccharides is difficult to study because the insoluble substrate is not amenable to straightforward biochemical analysis and soluble intermediate oligosaccharide products are degraded fast and, therefore, difficult to detect. Thus, usually the only detectable products during degradation of cellulose are mono-, di-, and trisaccharides, which are typical end products.[13–16] This is the reason that there are only a few studies on the relation between the fine structure of cellulose and its biodegradability.[17–20] The effect of the variety of physical structures adopted by the cellulose molecules in its different crystalline forms on biodegradation was not yet investigated in detail. In this paper the effect of polymorphism on the biodegradability of cellulose, was evaluated. In addition, the relative importance of different structural parameters on the enzymatic

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hydrolysis was analyzed and the hydrolysis rate for each allomorph was established.

Experimental Part

Materials

It was used three kinds of cellulose samples: m*icrocrystalline cellulose* – AI - Avicel HP-101 (Fluka) (DP = 183); c*otton cellulose* – BI – Pakistan (DP = 3078); s*pruce dissolving pulp* – EI - Extranier F (Rayonier) (DP = 1458). Microcrystalline cellulose and spruce dissolving pulp were used without any purification. Cotton cellulose was extracted in a Soxhlet extractor with ethanol and benzene, for 8 hours. It was then boiled in 1% aqueous solution of sodium hydroxide for 6 hours, washed with distilled water, immersed in 1% acetic acid, washed with water, and finally dried in air. Three allomorph forms were prepared from spruce dissolving pulp: *cellulose I* (EI); *cellulose II* (EII) - was prepared from cellulose I by soaking it in 17.5% NaOH for 24 hours at 15°C, followed by washing thoroughly with distilled water and dried in air; *cellulose III* (EIII) - was prepared from cellulose I by soaking in organic amine (100% ethylendiamine) for 24 hours at room temperature, washed with anhydrous methanol and finally air-dried.

Method of Hydrolysis

The allomorphs were subjected to enzymatic hydrolyses using enzymes produced by fungus *Trichoderma reesei* (Merck). After preswelling of 0.5 g cellulosic substrate, in the recipient was added 16.5 mL of 1 M citrate buffer (pH 4.8) and 0.5 mL of enzyme solution. The flask was placed in a 50 °C incubator. Samples were withdrawn at different time periods, 2, 4, 6, 8 and 10h, centrifuged and the supernatant was refrigerated.

Degrees of Polymerization

Degrees of polymerization of cellulose (DP) were measured by the viscosity method in 0.5 mol Cuen.[21]

X-Ray Diffraction Method

X-Ray diffraction patterns of the samples were collected on a RIGAKU RINT 2500 apparatus (Japan), equipped with a transmission type goniometer using nickel-filtered, CuK_α radiation at 40kV. The resulting diffraction patterns exhibited peaks which were deconvoluted from a background scattering by using Lorenzian functions, while the diffraction pattern of an artificially amorphicized sample was approximated by a Gaussian functions curve fitting analysis.[22] The estimation of the crystallinity index (Cr.I.) of cellulose samples was determined by the Segal method, which consists in the estimation of the peaks intensity corresponding to crystalline and amorphous areas.[23] The average size of the crystallites, measured in the directions orthogonal to the (101), $(10\bar{1})$ and (002) plane, was calculated with the Scherrer equation.[24]

Results and Discussion

One of the major obstacles that have to be cleared for the full understanding of the enzymatic degradation of cellulose is the influence of parameters such as accessibility, crystallinity and supramolecular structure of the substrata. In this idea was initially study the enzymatic hydrolysis of celluloses with crystalline structure of cellulose I, but with different morphologies, such as, microcrystalline cellulose (AI), cotton cellulose (BI) and spruce dissolving pulp (EI). The kinetic data obtained from the enzymatic degradation shows that their supramolecular and morphological structure influences in a major way the reaction ratio. From Figure 1 can be notice that the greatest biodegradation ratio is encountered in the case of spruce dissolving pulp. This situation could be explained through the presence of a bigger accessible surface determined by a higher content in amorphous material. Microcrystalline cellulose is characterized by an intermediate value of enzymatic hydrolysis ratio, due to the fact that, although its structure contains

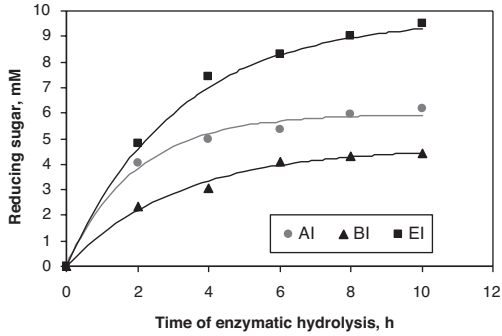

Figure 1.
Time-course of enzymatic hydrolysis of different types of cellulosic materials.

structural elements with the highest degree of order, these are of small dimensions and thus the accessible surface of the enzymatic complex is bigger. Cotton cellulose presents the slowest reaction ratio, because of the presence of a structure composed of grouped microfibrils, with compact texture, which makes difficult the action of the enzymatic complex toward the cellulosic substratum.

It can be concluded that in the enzymatic degradation taking place in heterogeneous conditions, the scission of bonds is controlled by the physical structure of cellulose, namely by the limited accessibility to the glucosidic bond within the structure of cellulose. In addition, it is known that the hydrolysis rate is directly proportional to the specific surface area of cellulose.

To increase the adsorption of cellulase to cellulose surface it is necessary to realise a pre-treatment of cellulose which will leads to changes in pore size distribution, so that more of the surface area within pores will become accessible to cellulases of a certain size. In native untreated cellulose only a small fraction of the pores are accessible to cellulose and for an enhancement of hydrolysis ratio of cellulose it is necessary to realize chemical modification of cellulose. To determine the influence of the modifications induced by the chemical treatments of cellulose activation upon the enzymatic hydrolysis it was selected the spruce dissolving pulp. It was obtained three allomorph forms of spruce dissolving pulp (EI, EII and EIII) and was evaluated the influence of the crystalline type of organization and of the allomorph type of cellulose, respectively, on the process of the enzymatic hydrolysis reaction.

From Figure 2 it can be observed the differences between the reaction ratios of enzymatic hydrolysis in the case of the

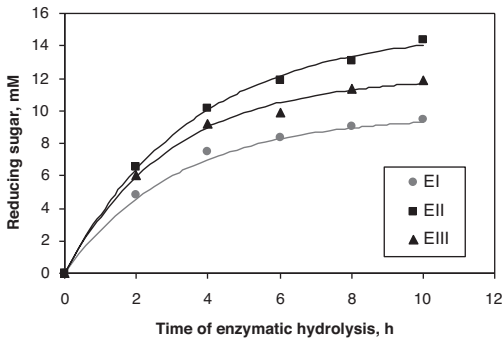

Figure 2.
The enzymatic degradation of allomorphic forms of microcrystalline cellulose.

polymorphic forms of spruce dissolving pulp and it is emphasized the fact that a strongly chemical modification of cellulasic substratum increase the hydrolyses ratio. Evidently, the cellulose with the less ordered structure is the most susceptible to the topochemical reaction of hydrolysis. The fact that the allomorphic form of cellulose III presents a higher reaction ratio than cellulose I is explained by the differences which appear in the intra- and intermolecular bonds of supramolecular structure of each type of allomorph.

The values obtained for the kinetic parameters Km and Vmax are presented in Table 1. In the case of allomorphic forms of spruce dissolving pulp, the Michaelis – Menten type of kinetics was followed.[25–27]

The Km parameter represents the affinity of the enzymatic complex employed toward allomorphic forms of spruce dissolving pulp and this decreases in the order: EIII > EI > EII. The maximal rate can be considered proportional to the reactivity of the substratum, so the generally order in relation to the reactivity of allomorphic forms is: EII > E II > EI, determined by the modifications which take place in the supramolecular structure of the cellulose allomorphs.

Table 1.
The kinetic parameters of polymorphic forms of cellulose enzymatic hydrolysed.

Sample	Michaels' constant, Km, mM	The initial maximum rate, mM/h
EI	362.42 ± 0.06	8.66 ± 0.67
EII	530.11 ± 0.07	12.08 ± 0.33
EIII	350.62 ± 0.35	6.33 ± 0.89

The diffractograms of the initial samples (EI) and of the sample enzimatically treated for 10 hours (EI5) are presented in Figures 3 and 4. It can be observed that residue resulting from enzymatic hydrolysis

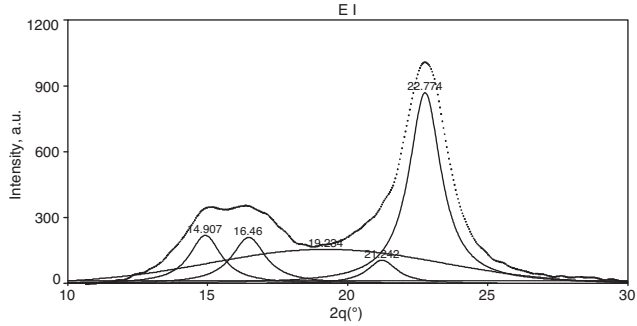

Figure 3.
The X-ray diffractogram of spruce dissolving pulp (EI).

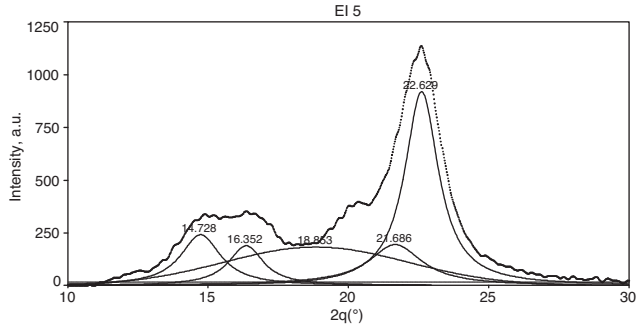

Figure 4.
The X-ray diffractogram of EI enzymatically degraded for 10 hours (EI5).

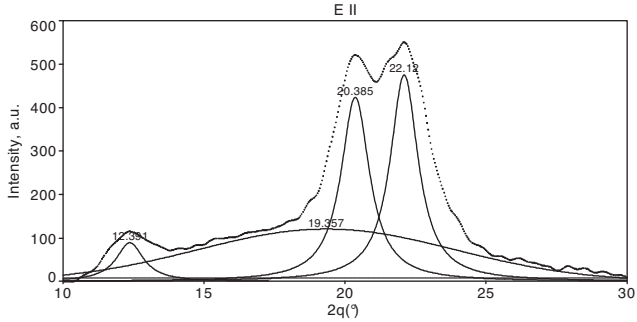

Figure 5.
The X-ray diffractogram of cellulose II obtained from spruce dissolving pulp (EII).

reaction present a diffractogram that do not modify their shape characteristic to the crystalline form of cellulose I. The diffractogram of residues obtained after the enzymatic hydrolysis of allomorphic form of cellulose II, performed after 10 hours of enzimatically degraded (EII5), indicate a maintaining of the crystalline form of organization like the initial sample – EII (Figures 5, 6).

A special behavior appears in the case of cellulose III, where a partial reversibility to the cellulose I form, during the enzymatic hydrolysis, can be observed (Figures 7, 8).

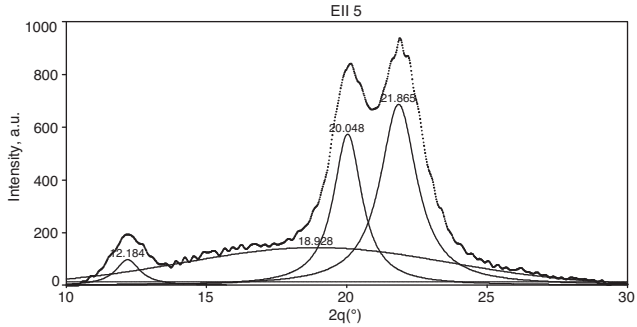

Figure 6.
The X-ray diffractogram of EII enzymatically degraded for 10 hours (EII5).

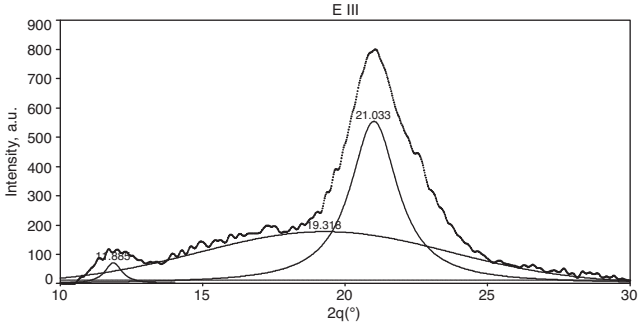

Figure 7.
The X-ray diffractogram of cellulose III obtained from spruce dissolving pulp (EIII).

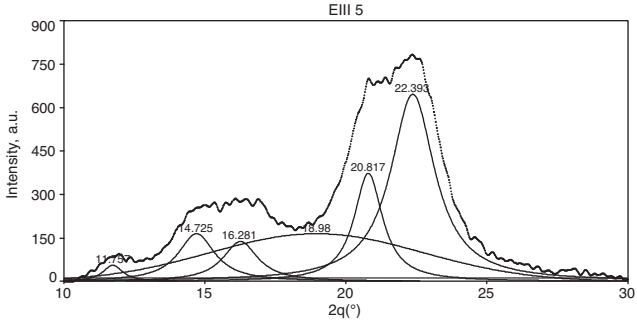

Figure 8.
The X-ray diffractogram of EIII enzymatically degraded for 10 hours (EIII5).

Thus, in the diffractograms of the enzymatically hydrolyzed residues appear the reflection of (101), (10$\bar{1}$) and (021) planes, to values characteristic of cellulose I. Also, if the reflection of (101) plane of cellulose III remain at approximately the same value for the treated (EIII5) or untreated (EIII) samples, in the case of the reflection of (002) plane this appears at a much higher value of the Bragg angle for the case of hydrolysed sample then the initial sample, similar to those characteristic to cellulose I.

These values confirm the partial return of cellulose III to cellulose I and leads us to assume that there is a juxtaposition of the reflection of the (002) plane of cellulose III with that of cellulose I. An explanation of this partial reversion is the presence of the water-based medium and of the high temperature of the reaction (50 °C). The obtained cellulose still retains a certain disorganized state at the level of supramolecular structure, which makes it more accessible to the enzymatic attack.

Table 2 presents the crystallite dimensions, the values of the crystallinity index and the degree of polymerization for the initial cellulose samples and for those enzymatically degraded for 6 and 10 hours, respectively.

The slight increase of the degree of crystallinity of the cellulosic material during the process of the enzymatic hydrolysis reaction was explained through the attack on the macromolecular chains placed in accessible regions. It was finding that during the reaction, the size of crystallites in the (101) and (10$\bar{1}$) direction remains approximately around the same value, while in the (002) direction the decrease is much more significant, compared to the initial sample. The values of the degree of polymerization obtained for initial cellulose and for the residues obtained after different time

Table 2.
Crystallite dimensions, crystallinity index and the degree of polymerization of the cellulosic samples enzymatically degraded.

Sample	Crystallite dimension, Å			Cr.I., %	DP
	D (101)	D (10-1)	D (002)		
EI	32.99	47.85	38.70	65.47	1458
EI3	32.72	46.89	38.50	68.04	999
EI5	30.56	44.56	34.54	70.80	957
EII	46.55	42.51	39.94	57.14	1116
EII3	46.07	41.02	38.51	59.65	725
EII5	45.88	38.47	38.03	60.53	660
EIII	49.17	–	40.56	47.78	1179
EII3	48.94	–	39.70	50.68	884
EIII5	47.07	–	32.36	54.02	808

periods of enzymatic degradation show an important modification which takes place in the first two hours of hydrolysis. After that, the decrease of DP takes place more slowly.

Conclusion

The heterogeneous enzymatic hydrolysis of cellulose proves to be adequate for investigation of the influence of the modifications induced by chemical treatments of cellulose upon the development of the enzymatic hydrolysis process. The study of X-ray diffractograms of residues resulting from enzymatic hydrolysis show the fact that after biodegradation, the crystalline structure of allomorphic forms I and II does not suffer significant modifications, while for the polymorphic form of cellulose III, a partial reversion to the crystalline structure of cellulose I, was observed. The values of the degrees of crystallinity for all the allomorphic forms enzymatically degraded indicate a slight increase during the process. The degree of polymerization of the cellulosic substrata enzymatically hydrolyzed decrease during the development of the hydrolysis process.

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