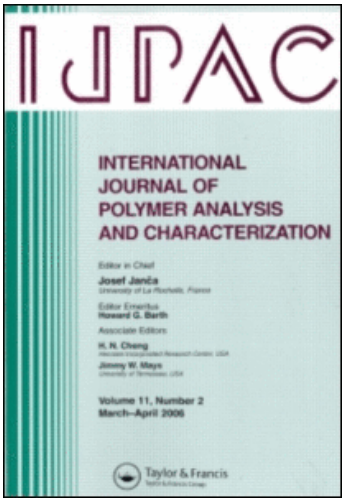

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Biotransformation of Cellulose: GPC Studies†

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We are interested in developing a new manufacturing process for cellulosic fiber based on biotransformation of cellulose to make it soluble in NaOH solutions. The enzymes used are cellulases extracted from *Aspergillus niger* by deep fermentation of the mycelium. The effect of the biotransformation process conditions on cellulose molecular structure and solubility in NaOH solutions was investigated, the overall objective being to optimize the process. The biotransformation process involves partial depolymerization of the cellulose with associated changes in the physical and chemical parameters of the polymer. The changes were determined by gel permeation chromatography (GPC). The effect of biotransformation parameters such as enzyme concentration, temperature and time of enzymatic action on the molecular properties of cellulose were studied. The effect of pretreatment methods such as mechanical or radiation were also determined.

Keywords: Biotransformed cellulose, cellulases, cellulose solubility, gel permeation

INTRODUCTION

To help eliminate toxic wastes, there is need to develop adequate biological methods based, among others, on the use of industrial scale application of microorganisms.[1] The chemical fiber industry presents numerous hazards to the environment, the greatest perhaps being those that are associated with the manufacturing of fibers from regenerated cellulose by the viscose

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method. Carbon disulfide and hydrogen sulfide, which are produced in the process, are both examples of toxic waste. Methods of eliminating the viscose process are being examined, one of which is the use of *N*-methyl morpholine oxide as a solvent for cellulose to transform cellulose into cellulose carbamate that could be further processed without the use of carbon disulfide,[2] or to obtain a form of cellulose that would be directly soluble in aqueous solutions of sodium hydroxide.[3] These two latter directions have been recently investigated at the Institute of Chemical Fibres, Lódź, Poland.

The possibility of obtaining a form of cellulose that would be soluble in solutions of NaOH seems particularly attractive. Such cellulose can be obtained through biotransformation of standard cellulose with the use of cellulases belonging in the group of glycoside hydrolases.[3–5] The biotransformation of cellulose pulps requires suitable pretreatment for opening of cellulose capillary system for cellulase penetration. Some pretreatment methods such as oxidation, radiation, mechanical method, *etc.* are commonly used.

Enzymatic transformation of cellulose involves a rather complex process in which the average molecular weight is reduced and other molecular parameters of the polymer are changed. At the same time, the cellulose activation phenomenon occurs.[3–5] Biotransformation process, which leads to obtaining fibrogenic cellulose of improved reactivity is, at the same time undoubtedly connected with the reduced strength of intermolecular hydrogen bonding believed to be chiefly responsible for the insolubility of cellulose.

Cellulases degrade cellulose by reducing its molecular weight without changing its chemical composition and without undesirable by-products. In the biotransformation process, however, use is made not of individual enzymes but of enzyme complexes consisting of various enzymes of different properties and range of activity. The degradation process is initiated by endoglucanases which break up the cellulose chain. This process increases the number of nonreducible ends which are susceptible to other enzymes included in the complex, such as exoglucanases and cellobiohydrolases, the former being capable of releasing glucose from the nonreducible chain ends of cellulose, while the latter can similarly reduce cellobiose. The remaining group of enzymes included in the complex are β-glucosidases. They do not affect cellulose, but they can decompose cellobiose and other oligosaccharides into glucose. The action of the enzymatic complex on cellulose is represented in Figure 1.[6]

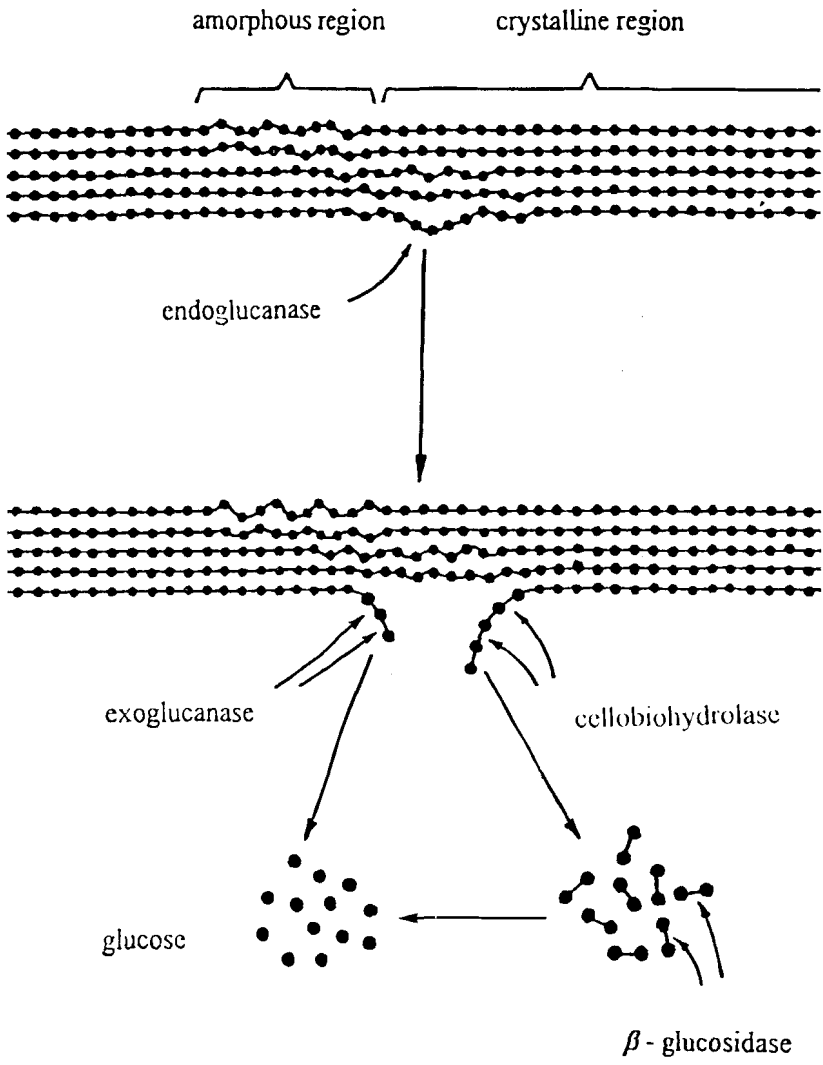

FIGURE 1 Action of enzymatic complex on cellulose.[6]

Most of the cellulases are derived from the strain *Trichoderma reesei*,[7] but as shown in ref.[8] the strain *Aspergillus niger* supplies the best enzyme complex for the biotransformation of cellulose.

The biotransformation of cellulose is a heterogeneous process taking place in an aqueous medium, the latter being a solvent for the enzyme

complex which catalyzes the changes.[9] It is, therefore, obvious that the rate of changes catalyzed by the cellulases will depend on the availability of the susceptible macromolecular chains. This availability is dependent, however, on the macrostructure of cellulose. Samples of a compact macrostructure are less susceptible to the enzymes than the fiber form of the samples. For the biotransformation process, the character of the capillary properties of the sample is important since contact of an enzyme molecule with an inner portion of cellulose is possible only if the pores of the capillaries are greater than the size of the enzyme molecule.[10]

In experiments with the biotransformation of cellulose, it was found that both the amorphous and the crystalline areas of the polymer could be made more susceptible to the enzymes if the commercial cellulose were pretreated using different methods. The increased availability of the macromolecular chains is a result of the looser structure and increased susceptible inner area of the cellulose. Therefore mechanical pretreatment increases the rate and effectiveness of the process of cellulose biotransformation.[11]

The changes occuring in the biotransformation of cellulose at the molecular level result not only in a reduction of the average molecular weight, but also in a different molecular weight distribution. The objective of this paper is to report the changes associated with the biotransformation of cellulose by determining the molecular parameters of untreated cellulose and of biotransformed cellulose, and to find out using gel permeation chromatography (GPC) how the molecular parameters are influenced by the selected pretreatment methods.

EXPERIMENTAL

Materials

The materials used in the experimental part were cellulases extracted by deep fermentation from *Aspergillus niger* at the Institute of Technical Biochemistry of the Technical University of Lódź, and two types of cellulose pulps: spruce-wood cellulose types Fibrenier and beech-wood cellulose (Cellulose Corp., Świecie, Poland). The materials are characterized in Tables I and II.

TABLE I Properties of commercial cellulose pulp in the biotransformation process

Type of cellulose pulp	*Source*	*Producer*	*DP**	*CrI*† *(%)*	*WRV*‡ *(%)*
Fibrenier	Spruce	USA	756	74	62
Swiecie Co. Ltd.	Beech	Poland	737	71	65

*degree of polymerization
†index of crystallinity
‡water retention value

Pretreatment of Cellulose Pulp

The mechanical pretreatment of aqueous dispersion of cellulose samples with a cellulose-to-water ratio of 1:16 was carried out using a tearing machine (Wearner-Pfeiderer) at 20°C for 1h. Berol Visco 32 produced by Akzo Nobel Co. was used as the surfactant in concentration 0.5%.

The radiation of commercial cellulose was carried out at the Institute of Applied Radiation Chemistry, Technical University of Lódź. The sample of cellulose was placed in a tight-sealed plastic bag and subjected to γ-radiation from a ^{60}Co source. The applied total radiation dose was 6 kGy.

Biotransformation of Cellulose

Sample of cellulose pulp, after pretreatment, was treated with an enzyme solution of concentration from 1 to 3 CMC Units/mL in a 0.05 M acetate buffer at pH 4.8. The enzymatic reaction was carried out in a water bath, at

TABLE II Characteristic of cellulolytic enzymes (*A. niger* ferm. II B) used in the biotransformation process

Enzyme activities			
CMCA (U/mL)	FPA (U/mL)	β-gluc (U/mL)	CMC/FPA
9.0	0.8	59.9	11

CMCA: endo-1,4-β-glucanase activity using carboxymethylcellulose.
FPA: total cellulase activity using filter paper.
β-gluc: β-glucosidase (cellobiose) activity using salicin.

30–60°C for 2 to 24 h. Following the enzyme treatment the enzyme solution was separated on a Buchner filter-funnel.[12]

Analytical Methods

Average degree of polymerization of commercial samples was determined viscometrically in a solution of alkaline ferrous sodium tartarate solution (EWNN).[13] Index of crystallinity was determined by X-ray diffractometry on a Rigaku—Denki apparatus, Geigerflex.[14] Water retention value (WRV) was determined by a gravimetric method.[15] Activities of the cellulase complex (CMCA, FPA, β-glucosidase activities) were determined by a colorimetric method.[16] Determination of degree of solubility was carried out according to the procedure described in ref.[5]

Gel Permeation Chromatography

The samples of cellulose to be analyzed by GPC were prepared in accordance with the procedure developing by Ekmanis.[17] Cellulose was dissolved in DMAC/LiCl using the following steps: preswelling in water (to activate the capillary structure), repleacment of H_2O by DMAC using a microwave oven, and finally dissolving in DMAC/8%LiCl. Upon complete dissolution of cellulose, the solutions were diluted with DMAC to a concentration of ~0.05%. Prior to GPC, the sample solutions were filtered throught PTFE filter (2μm, Sartorius).

In the GPC analysis, the solvent DMAC/0.5%LiCl was used as a mobile phase. The solvent was vacuum filtered using PTFE filters (0.45μm, Sartorius).

The filtered solutions of cellulose were analyzed by means of a GPC system consisting of a pump HP 1050 (Hewlett-Packard), manual injector (Rheodyne Inc., model 7125). and a refractive index detector HP 1047A (Hewlett-Packard). The mobile phase DMAC/0.5%LiCl was pumped at a flow rate 0.8 mL/min. The column was PLgel Mixed B, 600mm long (Polymer Laboratories, Ltd.) operated at 80°C. The volume of injection was 70μL. Calibration was with polystyrene standards (M_w 3,200–7,100,000) (Polymer Laboratories, Ltd.) as shown in Figure 2. The data were collected and processed by PL Caliber GPC/SEC Software Version 5.1 (Polymer Laboratories, Ltd.). Weight and number average molecular weights (M_w and M_n) as well as degree of polymerization DP_w were calculated.

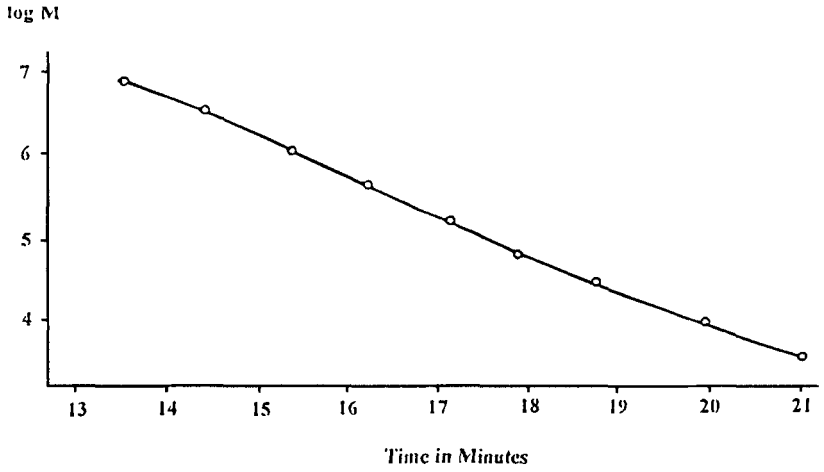

FIGURE 2 Calibration curve for polystyrene standards (Mw 3,200–7,100,000) in DMAC/0.5% LiCl (80°C). Curve fitting using 3rd-order polynomial.

DISCUSSION

The biotransformation of cellulose pulps being a complex phenomena of degradation and activation results in the improved solubility in alkali. To obtain the highest solubility of biotransformed cellulose required for technological applications, suitable pretreatment should be applied. The molecular weight distributions of Fibrenier pulp, also after appropriate pretreatment, is presented in Figure 3. The degradation effect of biotransformation process connected with its pretreatment is visible (curves 2 and 3). The solubility of the investigated samples in 9 wt % aqueous sodium hydroxide was also studied. The most effective pretreatment method combined with biotransformation seems to be the mechanical method resulting in obtaining cellulose with solubility degree of S_a not lower than 90%.

The action of cellulases is based, among other variables, on their capability to degrade the β-1,4 glycosidase bonds, reducing its average degree of polymerization. The character and range of changes in the molecular structures of cellulose are dependent on the enzyme type, concentration and reaction time. The process of biotransformation of cellulose type Fibrenier was carried out for 2, 4, 6 and 24h at 50°C using enzyme concentrations from 1 to 3 CMC Units/mL. The effect of enzyme concentration on the bio-

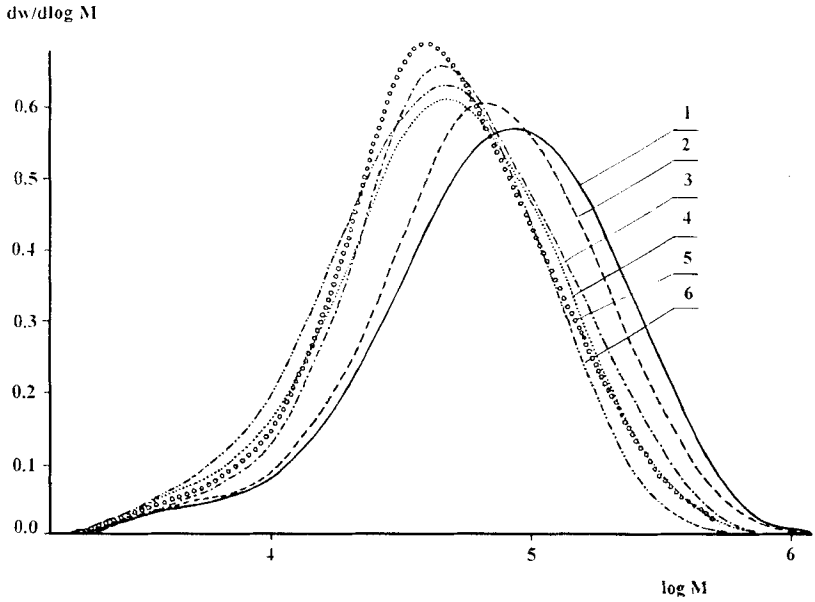

FIGURE 3 Molecular weight distributions of cellulose (Fibrenier) pulps: 1: commercial sample; 2: after mechanical pretreatment (initial); 3: after mechanical pretreatment and biotransformation; 4: after radiation pretreatment; 5: after radiation and mechanical pretreatment; 6: after radiation and mechanical pretreatment and biotransformation; 7: after radiation pretreatment and biotransformation

transformation process was judged by the changes in the molecular characteristic and degree of solubility of cellulose in a 9% aqueous sodium hydroxide solution. The results are presented in Table III and Figure 4.

On the basis of these data, enzyme concentration from 1 to 3 U/mL results in cellulose chain degradation, which is most intensive during the first two hours. The phenomenon is accounted for by the action of endoglucanase which degrades the cellulose macromolecule, significantly reducing the average degree of polymerization. With longer times of treatment of the degradation process is slowed down visibly (Fig. 4).

The results presented in Table III enabled the determination of the optimum enzyme concentration, which was found to be 2.0 CMC Units/mL for a cellulose content of 5 wt % in the cellulose and enzyme mixture. As it seen from Table III, the solubility of cellulose remains unchanged if treatment time is prolonged beyond 6 h which appears to be the optimum time of treatment under the adopted conditions.

TABLE III Effect of enzyme concentration on properties of biotransformed cellulose Fibrenier after mechanical pretreatment with Berol BV-32

Enzyme concentration (U/mL)	*Time of biotransformation (h)*	*Characteristic of cellulose*							
		M_n	M_w	DP_w	*PD*	*Content of DP fractions (%)*			*Solubility* S_a
		$\times 10^{-4}$	$\times 10^{-4}$			*<200*	*200–550*	*>550*	*(%)*
Initial sample	–	3.9	11.5	712	3.0	24	33	43	17
1.0	2	2.9	7.7	472	2.6	36	35	29	60
	4	2.7	7.3	449	2.7	41	35	24	63
	6	2.7	7.0	432	2.6	38	42	20	68
	24	2.3	5.9	366	2.6	46	34	20	83
2.0	2	2.8	7.3	450	2.7	37	42	21	70
	4	2.5	6.6	408	2.6	45	34	21	78
	6	2.4	6.4	396	2.7	42	41	17	89
	24	2.2	5.9	363	2.7	48	33	19	90
3.0	2	2.6	6.8	421	2.7	41	35	24	70
	4	2.3	6.4	397	2.8	44	34	22	76
	6	2.3	6.0	370	2.6	44	40	16	88
	24	2.2	5.4	335	2.5	46	40	14	90

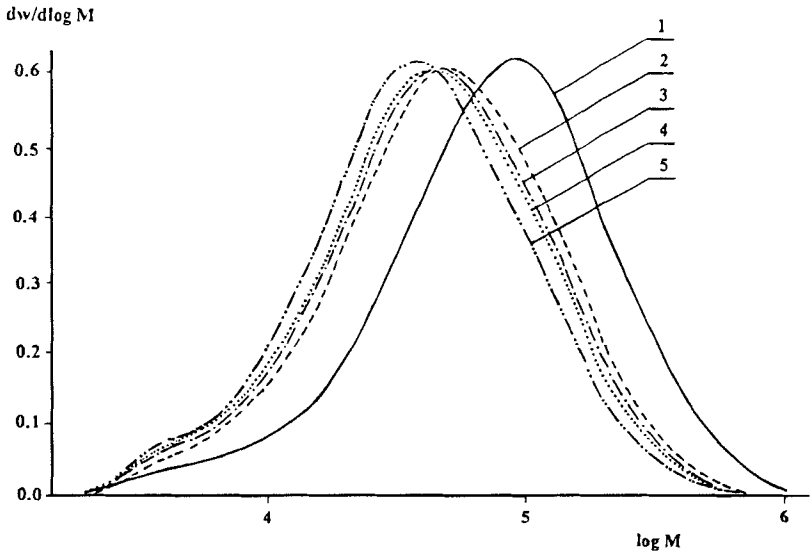

FIGURE 4 Molecular weight distribution of cellulose after mechanical pretreatment and biotransformation: 1: initial sample, 2: after 2 h of biotransformation, 3: after 4 h, 4: after 6 h, 5: after 24 h.

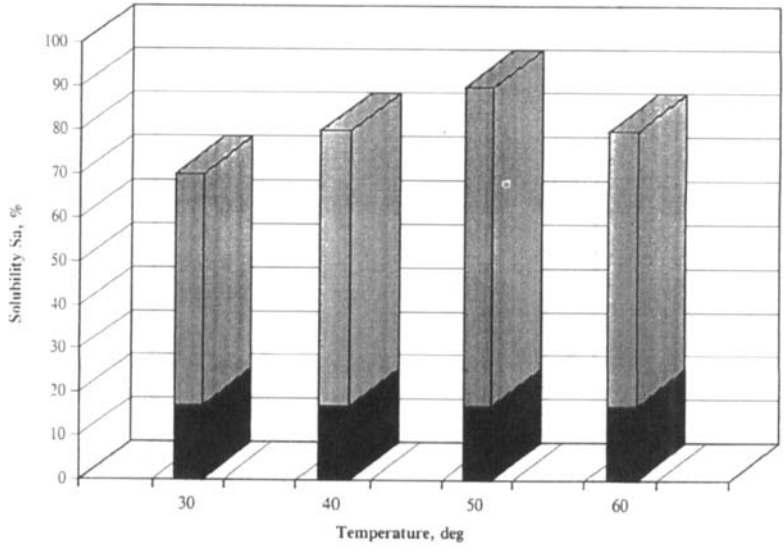

FIGURE 5 Effect of enzymatic treatment temperature on cellulose solubility; hatched: cellulose after biotransformation; solid: initial cellulose.

TABLE IV Effect of temperature on the molecular structure parametrs of cellulose Fibrenier after mechanical pre-treatment and biotransformation with cellulase concentration of 2.0 CMC U/mL

Biotransformation parameters		*Biotransformed cellulose properties*					
Temperature	*Time*	DP_w	*PD*	*Content of DP fractions (%)*			*Solubility* S_a *(%)*
(°C)	*(h)*			*<200*	*200–550*	*>550*	
Initial sample	–	677	3.5	23	42	35	17
30	6	433	2.4	31	42	27	70
40	6	395	2.3	34	43	23	80
50	6	389	2.4	36	40	24	90
60	6	426	2.4	33	41	26	80

Temperature is another important factor in the biotransformation process. This was investigated using similar conditions (cellulose concentration 5%, enzyme concentration 2 CMC Units/mL, time 6 h) at 30° to 60°C. The results are presented in Figure 5 and Table IV.

From data presented in Table IV, the maximum increase of cellulose solubility takes place if biotransformation process is carried out at 50°C. These important results from the technological point of view are shown in Figure 5. It can be seen that at the higher temperature (60°C), the action of the enzyme is slowed down (higher than for the temp. 50°C DP value and lower solubility S_a).

CONCLUSIONS

GPC was used to characterize molecular properties of cellulose pulp subjected to biotransformation by means of cellulolytic enzymes. As a result, the process of cellulose activation takes place as well as an increased solubility in aqueous solutions of alkali. Biotransformation is accompanied by changes on the molecular level by degradation of the polymer. The molecular parameters under examination are affected by conditions of both biotransformation and pretreatment of cellulose. It was found out that the solubility of biotransformated cellulose is greatly influenced by its mechanical pretreatment with the presence of surfactant. This pretreatment, however, does not involve considerable changes in molecular characteristics of cellulose. Based on the analysis of molecular parameters and solubility, the optimum conditions of biotransformation process were determined.

Acknowledgements

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