Structural Features of Native Cellulose Gels and Films from Their Susceptibility to Enzymic Attack

PIER LUIGI BELTRAME,[1,*] ERNESTINA DUBINI PAGLIA,[1] ALBERTO SEVES,[2] ELISABETTA PELLIZZONI,[2] and MARIA ROMANÒ[3]

[1]Dipartimento di Chimica Fisica ed Elettrochimica, Università di Milano, Milan, Italy, [2]Stazione Sperimentale per la Cellulosa, Carta e Fibre Tessili Vegetali ed Artificiali, Milan, Italy, and [3]Stazione Sperimentale per la Seta, Milan, Italy

SYNOPSIS

The reaction of enzymic hydrolysis has been used as a probe to evidence the different structural features of bacterial native cellulose gels and films synthesized from different carbon sources. The gels were found to be more hydrolyzable than were the films, both in terms of reaction extent and of initial reaction rate, by factors increasing with temperature. For instance, a cellulose gel synthesized from glucose showed at 50°C a hydrolysis yield twice as much as that of the corresponding film, thus revealing, in the former case, a higher level of substrate accessibility and enzyme penetrability. It has been suggested that the increase of gel accessibility with temperature can be associated with a corresponding lowering of the amount of structured water close to the polymer chains in the gel. The significant decrease of susceptibility to enzymic attack observed in going from the cellulose film obtained from glucose to that obtained from xylose has been related to the markedly lower value of specific surface area estimated in the latter case. Likely, in the film obtained from xylose, densely packed microfibrils occur that are scarcely accessible to enzyme. In some cases, the reaction progress has been followed by SEM analysis. Microcrystalline cellulose has been also considered for comparison.

INTRODUCTION

The kinetics of thermal *cis–trans* isomerization of Chrisophenine dispersed in a bacterial native cellulosic film has been recently determined.[1] The film was prepared by dehydration of a cellulose gel synthesized by *Acetobacter xylinum* using glucose as the carbon source. The kinetic measurements showed that the amorphous region of the matrix behaves as a glassy polymer in the temperature range explored (36–66°C) provided that the equilibrium relative humidity is maintained. Furthermore, the distribution of free volume in the polymer was found to be less homogeneous, and its extent likely smaller than in cellulose II. Thus, as previously observed,[2–8] isomerization of dispersed azo dyes proved to be an useful probe for characterizing the microstructure of the amorphous phase of the dispersing medium.

When the cellulose film is immersed in water, marked structural modifications intervene as water, acting as a plasticizer, lowers the glass transition temperature of the material and the glassy region is transformed into a rubber phase. In this case, useful structural indications about the polymer can be achieved considering as a probe another reaction strongly depending on the morphological features of cellulose substrate, namely, enzymic hydrolysis. In fact, the susceptibility of cellulose to enzymic degradation is profoundly influenced by any structural feature of the fiber that can limit its accessibility or the diffusion of cellulolytic enzymes in close proximity to it. The most favorable situation in terms of substrate accessibility and enzyme penetrability is likely achieved in the cellulose gel, where multiple layers of water significantly hinder chain association and hydrogen-bond formation.

On the basis of the above considerations, it

* To whom correspondence should be addressed.

Journal of Applied Polymer Science, Vol. 44, 2095–2101 (1992)
 CCC 0021-8995/92/122095-07$04.00

seemed interesting to compare the susceptibility to enzymic attack of cellulose films and gels, in order to gain indications of their different structural features. Hence, a kinetic study has been carried out on the enzymic hydrolysis of the two types of substrate and, for comparison, of a microcrystalline cellulose (Avicel). For the synthesis of bacterial native cellulose, two different carbon sources have been considered, namely, glucose and xylose.

MATERIALS AND METHODS

Substrates

The following substrates were considered:

(a) Cellulose Gels

Bacterial native cellulose was obtained from a culture of *Acetobacter xylinum* as described by Romanò et al.[9] The cellulose gels, prepared in proper sterile containers (d = 20 mm), were obtained after at least 7–10 days growth using as the carbon source glucose (gel–XNC/G) and xylose (gel–XNC/X), respectively. They were separated from the culture medium and bacteria by washing with 2% sodium hydroxide at 70°C, until the turbidity due to bacteria disappeared, and, subsequently, with distilled water up to pH 7.0.

(b) Cellulose Films

The samples gel–XNC/G and gel–XNC/X were dehydrated by evaporation at room temperature in order to obtain the change to film. In this stage, the multiple layers of water present in the gels disappear, causing a closer chain association and irreversible formation of hydrogen bonds between hydroxyl groups.[10] Films XNC/G and XNC/X, respectively, with a thickness of 15–20 μm, were so-obtained.

(c) Microcrystalline Cellulose (Avicel, Merck)

Substrate characterization.

- Dry cellulose content: The gel or film samples, allowed to drain, were weighed and then dried at 105 ± 2°C up to a constant weight. The dry cellulose content was calculated as $(w_2/w_1)\cdot 100$, where w_1 and w_2 are the weights of the sample before and after drying, respectively. The following values were obtained: 0.95 and 0.28% for gels XNC/G and XNC/X, respectively; 87.88 and 95.16% for films XNC/G and XNC/X, respectively; and 94.19% for Avicel.
- Water retention value (WRV): 0.5 g of film samples was dispersed in 2-propanol for 2 min. The excess alcohol was eliminated by filtration under vacuum. The samples were then centrifuged for 40 min at 4000 rpm, weighed, dried for 16 h at 105 ± 2°C, and weighed again. The water retention value was calculated as

$$\text{WRV}\ (\%) = \frac{w_w - w_d}{0.786\ w_d} \cdot 100$$

  where w_w and w_d are the weights of wet and dried samples, respectively, and 0.786 is the specific constant for 2-propanol. The following values were obtained: 5.99% for film XNC/G and 0.51% for film XNC/X.
- Degree of polymerization (DP_w): It was measured viscosimetrically by the Sapped method[11] and was found to be 1850 and 1750 for celluloses XNC/G and XNC/X, respectively.
- Sugar content: Gas chromatographic analysis (Hewlett-Packard 5890) of cellulose synthesized with glucose as the carbon source showed 94.1% of glucose and 5.9% of other sugars. For cellulose synthesized from xylose, values of 80.0% of glucose and 20.0% of other sugars were obtained.

Enzymes. The enzymes were commercial cellulase [complex Endoglucanase (E_1) + Exoglucanase (E_2)] and cellobiase (E_3) (Novo Industri) with declared activity of 1500 NCU/g and 250 CBU/g, respectively.

Kinetic runs. The substrate (pieces of gel or film or powders) and the enzyme were weighed, put in 0.1 M sodium acetate buffer (pH 4.8), and mixed in a thermostated reactor, under mechanical stirring (450 rpm). The temperature was kept constant to within 0.1°C. The hydrolysis reaction course was determined with time at constant temperature in the range 30–50°C. Samples (5 mL), withdrawn from reactor at different times (20 min–72 h), were filtered through a 0.45 μm Millipore filter and analyzed for glucose determination.

All the experiments were carried out at a substrate concentration of 2 mg/mL in a sodium acetate buffer. The concentration of complex ($E_1 + E_2$) was 0.6 mg/mL, and that of E_3, 0.06 mg/mL.

Reducing sugars analysis. The reducing sugars produced in the enzymic hydrolysis of the cellulose samples were spectrophotometrically deter-

mined with Nelson Somogyi's method,[12] using glucose for calibration.

Wide-Angle X-ray Scattering. The WAXS data were obtained by a Siemens D-500 diffractometer, with a Siemens FK 60-10, 2000 W Cu tube. The samples were mounted on a carrier for specimen spinning with a rotational speed of 30 rpm.

Crystallinity Index from Infrared Spectra. Infrared spectra of cellulosic substrates were obtained with a Perkin-Elmer 983 in the 850–1500 cm^{-1} region. For measuring crystallinity, the infrared ratio of absorption at 1429 cm^{-1} (CH_2 scissoring motion)[13,14] and 893 cm^{-1} (vibrational mode involving C_1 of β-linked glucose)[13] was used.[15]

Scanning Electron Microscope (SEM) Analysis. The samples were hydrolyzed under the conditions previously reported. Temperature of incubation was 50°C. After 2 or 24 h of incubation, the enzymes were inactivated at 100°C for 20 min and the samples preserved at 4°C. Drops of the aqueous suspension of cellulose were deposited on a stub for SEM and dehydrated by evaporation. The gel cellulose was previously fixed by 3% glutaraldehyde solution in phosphate buffer (pH 7) for 3 h, then dehydrated by the critical point drying (CPD) method. The resulting cellulose samples were shadowed with gold and then examined at Philips SEM 515 (accelerating voltage 10–15 kV).

RESULTS AND DISCUSSION

The general sequence of enzymic reactions involves a direct physical contact between enzyme and substrate to form the initial complex. Cellulose, as a substrate, is a water-insoluble compound of great structural complexity, for which the complex formation can be achieved only by the difficult diffusion of enzyme to the susceptible sites on molecular surfaces within the accessible fiber. Thus, the susceptibility to enzymic attack strongly depends on the structural features of cellulose, and its value, in terms of reaction rate and extent, can mark such features.

In this study, the cellulosic substrates considered consist of gels or solid films of bacterial native cellulose, synthesized by *Acetobacter xylinum* using glucose or xylose as the carbon source and of a microcrystalline cellulose sample chosen for comparison.

Notwithstanding that the degree of polymerization can be considered of limited significance in determining the susceptibility of cellulose to enzymic hydrolysis, as the employed cellulases hydrolyze the polymer randomly along the length of the molecules, DP_w was maintained at a value around 1800 for both films. On the other hand, the sugar content of the substrates was somewhat different depending on the different carbon source employed for the synthesis of bacterial native cellulose. The cellulose grown with xylose was found to be poorer in glucose (80.0%) than that grown with this latter monose (94.1%).

As regards the structure of the gels as obtained from *Acetobacter xylinum,* various opinions have been given.[16] Colvin and Leppard suggested that the nascent fibril has a dense core surrounded by a sheath of amorphous gel[17]; Brown et al. reported that the nascent fibril is composed of bundles of chains in the tactoidal and noncrystalline phase[18]; and Kai and Koseki suggested that the nascent fibril is in the amorphous state, but the cellulose chains maintain a specific correlation with each other.[19] In the X-ray diffraction analysis, the lattice structure of films XNC/G and XNC/X here considered showed the typical diagram of native cellulose (cellulose I).[20]

The substrates have been subjected to enzymic hydrolysis in the presence of cellobiase activity (E_3) to reduce the inhibition phenomena due to cellobiose production during the degradation. The reactions, carried out in the temperature range 30–50°C, showed the time courses depicted in Figures 1–3. In every case, the glucose production reached maximum values within 48 h. The corresponding saccharification values are reported in Table I, while Table II collects the values of the initial rates of hydrolysis in the various cases.

As shown in Table I, in the case of sample gel–XNC/G, the maximum yield of reaction regularly increases with temperature, but this does not occur for the corresponding dried sample film–XNC/G, for which a substantially constant value around 44% has been observed. While a constant behavior at the longest time is expected for the same substrate regardless of temperature, the above-mentioned increase of yield has to be justified. It can be suggested that such an increase corresponds to a lowering of the amount of structured water close to the polymer chain in the gel. Likely, this lowering that results in enhancing the substrate accessibility and, consequently, its susceptibility to enzymic attack is progressively favored by the higher temperatures.

As expected, at every temperature, and mostly at

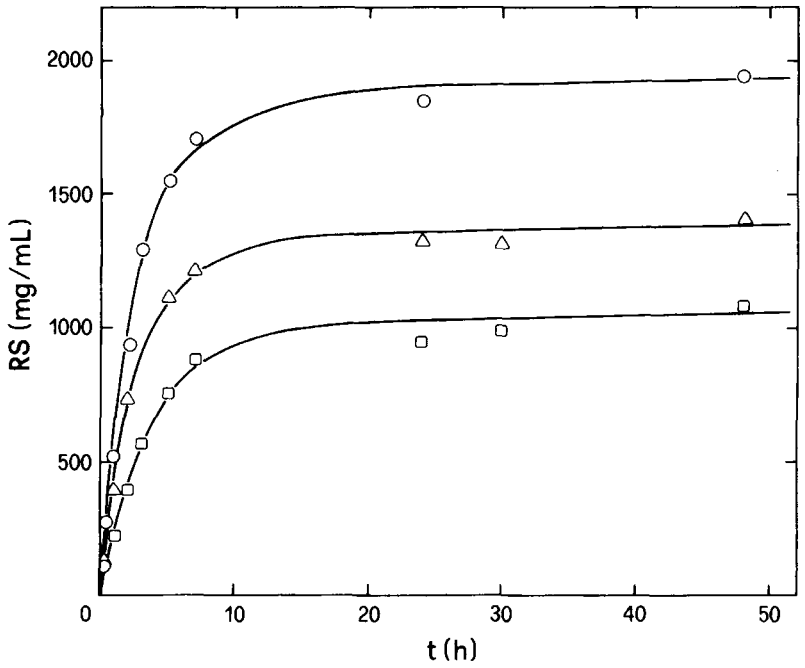

Figure 1 Time courses of enzymic hydrolysis of the bacterial native cellulose gel obtained using glucose as the carbon source (sample gel–XNC/G). RS = reducing sugars as glucose; (○) 50°C; (△) 40°C; (□) 30°C.

the highest one, the gels appeared to be more hydrolyzable than did the corresponding films; in particular, the gap is evident at 40°C in the case of cellulose grown with xylose, for which the value of maximum saccharification of gel is higher by a factor as high as 3.9 than in the case of film. On the other hand, the increase of the susceptibility to hydrolysis of cellulose grown with glucose, on going from film to gel at the same temperature, is somewhat less marked, namely, by a factor of 1.5. As expected, the Avicel sample was found to be the least accessible substrate, with a saccharification value equal to 15% of that of cellulose gel from glucose.

The differences in saccharification between films

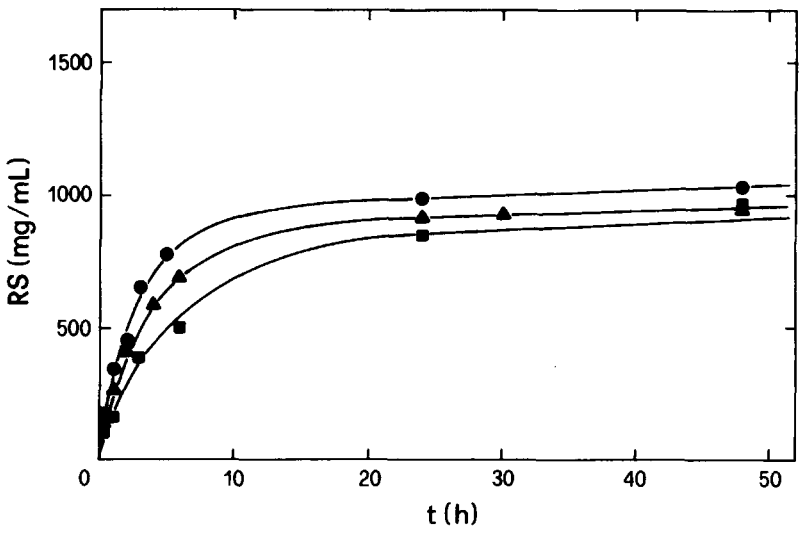

Figure 2 Time courses of enzymic hydrolysis of the bacterial native cellulose dehydrated gel obtained using glucose as the carbon source (sample film–XNC/G). RS = reducing sugars as glucose; (●) 50°C; (▲) 40°C; (■) 30°C.

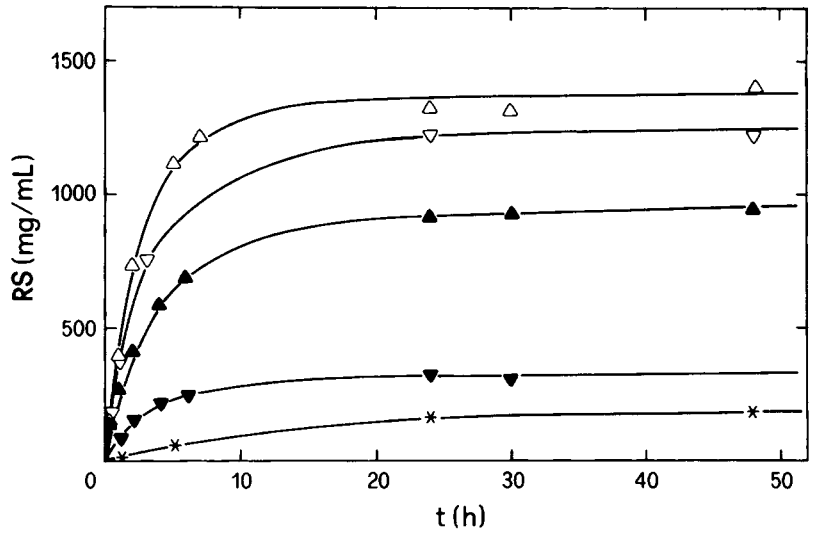

Figure 3 Time courses of enzymic hydrolysis at 40°C of the various cellulosic substrates considered. RS = reducing sugars as glucose; (△) sample gel–XNC/G; (▽) gel–XNC/X; (▲) film–XNC/G; (▼) film–XNC/X; (*) Avicel.

and gels are much less than those observed considering various regenerated cellulose films of normal and dry-cast gels prepared from viscose solutions.[21] This suggests that gels XNC/G and XNC/X are characterized by a molecular order much higher than that present in gels obtained from viscose solutions.

Some observations need to be made about the difference found at 40°C between the maximum saccharification values relative to the two samples gel–XNC/G and gel–XNC/X obtained from glucose and xylose, respectively. Likely, the two gels present similar accessibilities and penetrability and, hence, similar susceptibility to enzymic attack, and the above discrepancy can be merely ascribed to the different glucose content. In fact, taking into account this factor, comparable saccharification values can be obtained in the two cases. On the other hand, this explanation cannot hold for films XNC/G and XNC/X, as in this case the gap between the maximum saccharification values is too high, by a factor of about 3. Thus, marked structural modifications have to be involved on going from the former substrate to the latter one. This is confirmed by the strongly different water retention values obtained as estimates of the specific surface area of the two substrates (5.99 and 0.51 for film–XNC/G and film–XNC/X, respectively). Probably, the extent of packing of the microfibrils in the film obtained from xylose is much larger than that occurring in the film obtained from glucose.

It must be noticed that infrared measurements gave a higher value of crystallinity index[15] for film–

Table I Values of the Saccharification of Cellulosic Samples Reached by Enzymic Hydrolysis after 48 h at Various Temperatures (C_s^0 = 2 mg/mL; $C_{E_1+E_2}^0$ = 0.6 mg/mL; $C_{E_3}^0$ = 0.06 mg/mL)

	Saccharification (%)[a]		
Sample	30°C	40°C	50°C
Gel–XNC/G	48.8	63.4	87.5
Gel–XNC/X		54.7	
Film–XNC/G	43.7	42.8	46.2
Film–XNC/X		14.0	
Avicel		9.4	

[a] Calculated as [(mg reducing sugars as glucose formed after 48 h reaction)/(mg cellulose)] × (162/180) × 100.

Table II Values of the Initial Rates[a] of Saccharification of Cellulosic Samples by Enzymic Hydrolysis[b] at Various Temperatures

	Initial Rate (mg glucose/mL h)		
Samples	30°C	40°C	50°C
Gel–XNC/G	0.221	0.392	0.519
Gel–XNC/X		0.365	
Film–XNC/G	0.162	0.267	0.348
Film–XNC/X		0.083	
Avicel		0.008	

[a] Calculated at 1 h.
[b] For conditions, see Table I.

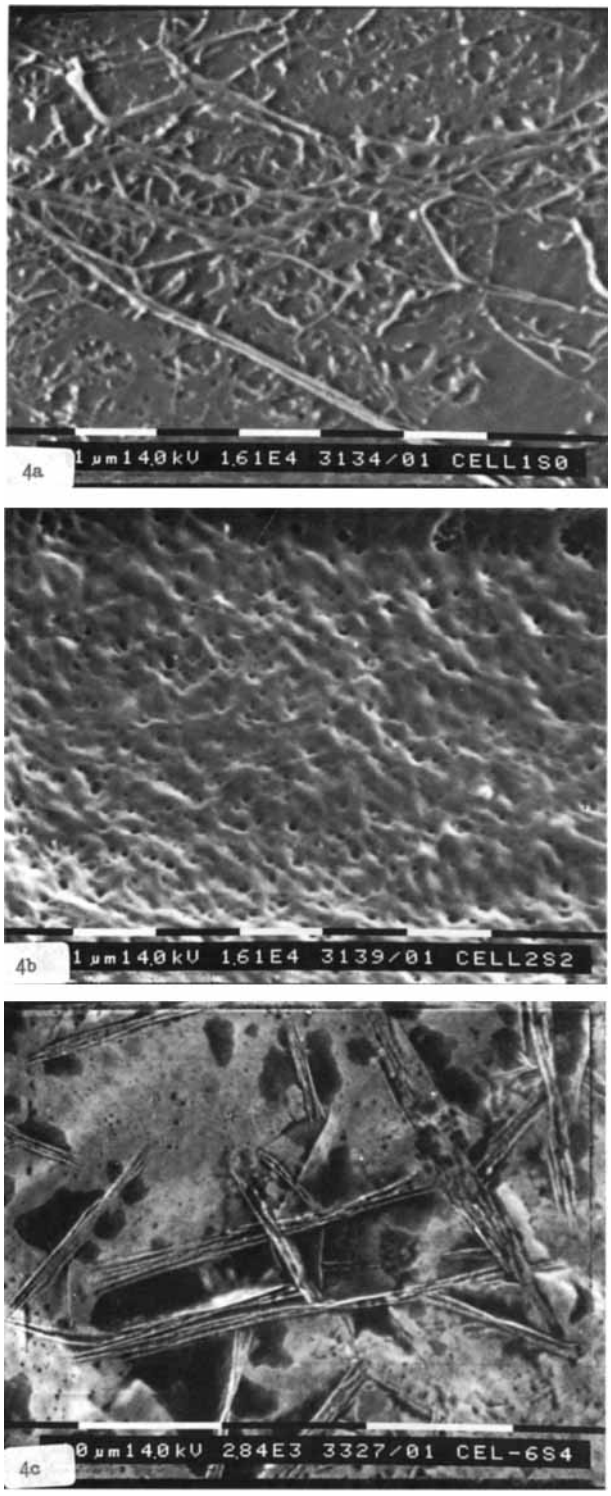

Figure 4 Scanning electron microscope analysis of sample film–XNC/G after different times of enzymic hydrolysis at 40°C: (a) $t = 0$; (b) $t = 2$ h; (c) $t = 24$ h.

XNC/G than for film–XNC/X (3.84 vs. 1.54). As the hydrolyzability is higher in the former case, it must be inferred that crystallinity plays no role in determining it, as reported for regenerated cellulose II films.[21] However, the high value of the crystallinity index for film–XNC/G might be ascribed merely to a larger amount of smaller crystallites rather to a greater extent of crystallinity itself.

The values of the initial rates of saccharification in Table II are consistent with the above considerations drawn from the maximum reaction extents. In particular, such kinetic results confirm the higher reactivity of the gels with respect to the films in the whole temperature range considered. Furthermore, they confirm the higher reactivity gap between the two films obtained with different carbon sources in comparison with the gap of the corresponding gels. Also from a kinetic point of view, the microcrystalline cellulose exhibited a much lower susceptibility to hydrolytic attack.

The observed dependence on temperature of the initial rates appears somewhat higher for the gel than for the film, thus suggesting a higher activation energy value in the former case. Likely, entropic factors can more than compensate for this difference in activation energy, resulting in a higher reaction rate for cellulose gel.

A scanning electron microscope analysis was performed on sample film–XNC/G at different reaction times (at 40°C), in order to gain a more detailed insight into the hydrolysis progress. From the inspection of Figure 4(a) and (b), it can be observed that the fibers initially on the film surface are completely destroyed after 2 h of incubation and that the structure below starts to be attacked.

After 24 h of reaction, the cellulose film pieces in the reactor were found to be flaked; clearly, this period was enough for the enzymes to penetrate and open on (or loosen up) internal surfaces and to more effectively attack individual chains or chain segments. Thus, the SEM analysis was carried out on the dried residual mucilages withdrawn from the reactor. This analysis [Fig. 4(c)] evidences the presence of microfibrils of cellulose, insensitive to enzymic attack, while the amorphous regions appear almost completely hydrolyzed.

The presence of microfibrils was easily observed, after the same incubation period, also considering sample film–XNC/X (Fig. 5), even if in this case the hydrolysis extent is significantly lower. As in Figure 4(c), the crystalline phase appears constituted by a multiplicity of microcrystals.

The SEM analysis was also employed on the cellulose gels dehydrated prior to metallization by the CPD method in order to maintain as far as possible their gel structure. The SEM analyses of the unhydrolyzed gel samples, obtained either from glucose

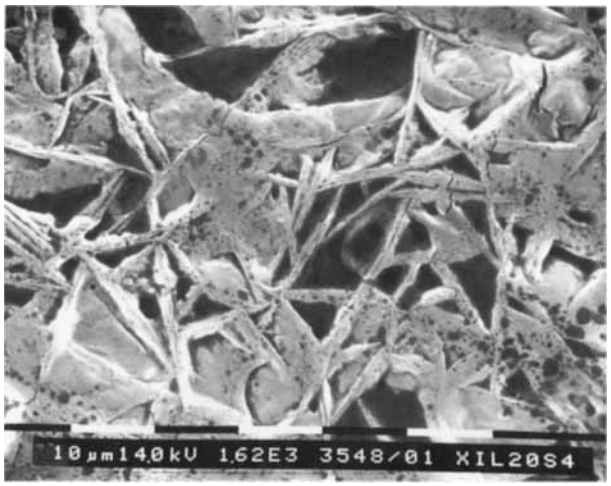

Figure 5 Scanning electron microscope analysis of sample film–XNC/X after 24 h of enzymic hydrolysis at 40°C.

or from xylose, were found to be quite similar.[9] As expected, the analysis of the gel sample from glucose at almost complete degradation did not reveal the occurrence of microfibrils.

REFERENCES

1. A. Seves, M. Romanò, E. Dubini Paglia, P. L. Beltrame, and B. Marcandalli, *J. Appl. Polym. Sci.*, to appear.
2. E. Dubini Paglia, P. L. Beltrame, B. Marcandalli, P. Carniti, A. Seves, and L. Vicini, *J. Appl. Polym. Sci.*, **31,** 1251 (1986).
3. P. L. Beltrame, E. Dubini Paglia, B. Marcandalli, P. Sadocco, and A. Seves, *J. Appl. Polym. Sci.*, **33,** 2965 (1987).
4. E. Dubini Paglia, P. L. Beltrame, B. Marcandalli, A. Seves, and L. Vicini, *J. Appl. Polym. Sci.*, **36,** 635 (1988).
5. B. Marcandalli, P. L. Beltrame, E. Dubini Paglia, and A. Seves, *Dyes Pigments,* **11,** 179 (1989).
6. P. L. Beltrame, E. Dubini Paglia, B. Marcandalli, and A. Seves, *J. Appl. Polym. Sci.*, **38,** 755 (1989).
7. E. Dubini Paglia, P. L. Beltrame, A. Seves, and G. Prati, *J. Soc. Dyers Colourists,* **105,** 107 (1989).
8. E. Dubini Paglia, P. L. Beltrame, B. Marcandalli, and A. Seves, *J. Appl. Polym. Sci.*, **41,** 765 (1990).
9. M. Romanò, G. Franzosi, A. Seves, and S. Sora, *Cellulose Chem. Technol.*, **23,** 217 (1989).
10. J. R. Colvin, *TAPPI,* **60,** 59 (1977).
11. Swedish Association of Pulp and Paper Engineers, Technical Communication, Determination of the Viscosity of Cellulose Cupriethylendiamine Solution, (CED)-CCA 28:57.
12. N. Nelson, *J. Biol. Chem.*, **153,** 375 (1944); M. Somogyi, *J. Biol. Chem.*, **195,** 19 (1952).
13. C. Y. Liang and R. H. Marchessault, *J. Polym. Sci.*, **39,** 269 (1959).
14. R. H. Marchessault and C. Y. Liang, *J. Polym. Sci.*, **43,** 71 (1960).
15. R. T. O'Connor, E. F. DuPré, and D. Mitchum, *Textile Res. J.*, **28,** 382 (1958).
16. A. Kai, *Makromol. Chem. Rapid Commun.*, **5,** 307 (1984).
17. J. R. Colvin and G. G. Leppard, *Can. J. Microbiol.*, **23,** 701 (1977).
18. C. H. Haigler, R. M. Brown, and M. Benziman, *Science,* **210,** 903 (1980).
19. A. Kai and T. Koseki, *Bull. Chem. Soc. Jpn.*, **57,** 1437 (1984).
20. E. M. Macchi, *Makromol. Chem.*, **191,** 2217 (1990).
21. R. H. Marchessault and J. St-Pierre, in *Future Sources of Organic Raw Materials-CHEMRAWN I,* L. E. St-Pierre and G. R. Brown, Conference Eds., Pergamon, New York, 1978, p. 613.

Received September 17, 1990
Accepted July 8, 1991