Influence of Substitution of Direct Dye Having Biphenylenebis(azo) Skeletal Structure on Nascent Cellulose Produced by *Acetobacter xylinum* [II]

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ABSTRACT: The influence of Direct Blue 14 and 53 dyes, both having biphenylenebis(azo) skeletal structure but different sulfonate groups substitution on the structure of the nascent microbial cellulose was examined. The product obtained from the *Acetobacter* culture in the presence of each dye is a characteristic dye–cellulose complex, and the dye molecule is included between the cellulose sheets in the complex corresponding to the $(1\bar{1}0)$ plane of microbial cellulose. Due to the inclusion of dyes between the cellulose sheets through hydrogen bonding or van der Waals forces, the hydrogen bonding between cellulose chains of microbial cellulose is hindered. The different position of sulfonate groups has no major influence on the two products except on the uniplanar orientation of the product. Celluloses regenerated from both products are cellulose II, but their fine structures are different from each other. © 1999 John Wiley & Sons, Inc. J Appl Polym Sci 71: 1007–1015, 1999

Key words: *Acetobacter xylinum;* direct blue 14; direct blue 53; dye–cellulose complex; regenerated cellulose II

INTRODUCTION

Acetobacter xylinum produces cellulose in a noncrystalline state outside the cell during metabolism.[1] But this cellulose crystallizes to cellulose I_α-rich structure through the development of hydrogen bondings between the adjacent cellulose chains.[2,3] This result supports the concept of cellulose I microfibrils crystallization without the direct involvement of living cells.[4,5] Using *A. xylinum,* a bacterium that synthesizes a highly crystalline ribbon of cellulose I, Kai et al.[3,6,7] and Haigler and coworkers.[8,9] have demonstrated that fluorescent brightening agents and direct dyes that bind to cellulose *in vivo* can separate glucan chain polymerization from microfibril crystallization. Brown et al.[9–11] reported that, judging from the electron microscopy of products from *Acetobacter* culture with direct dyes, dyes adhered to the surface of protofibril in a stack state from noncrystalline products, and, thus, this product forms cellulose I upon dye extraction. Kai et al.[7] found that products from the incubated medium with Direct Red 80 showed characteristic X-ray diffraction (XRD) diagrams indicating the formation of a crystalline complex, and this complex produces cellulose II after dye extraction. More recently, Kai et al. have regenerated a cellulose I_β-rich structure from a brightener complex.[3,12] On the other hand, cellulose regenerated from the complex obtained in the presence of Direct Red 28 (DR28) having biphenylenebis(azo) skeletal structure is similar to cellulose IVI, whereas that in the presence of Direct Blue 1 and

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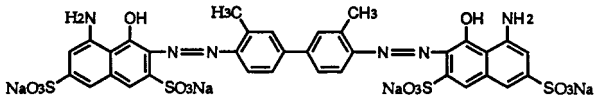
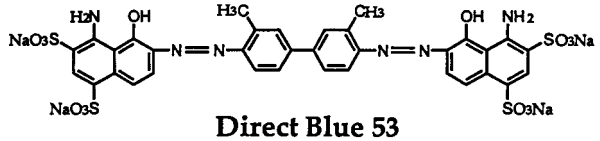


Direct Blue 14



Direct Blue 53

Figure 1 Chemical structure of direct dyes.

Direct Blue 15 (DB1 and DB15) having more two hydroxyl, two methoxy, and two sulfonate groups in the same skeletal structure of DR28 formed cellulose II.[13] Thus, direct dyes have different affinities for cellulose under experimental conditions, which would be affected by the kind, number, and position of substituents on the planar molecular backbone.[14]

In this study, in order to more clearly understand the structure of nascent microbial cellulose, the influence of Direct Blue 14 and Direct Blue 53 (DB14 and DB53), both having the same biphenylenebis(azo) skeletal structure on the nascent microbial cellulose was examined. The product from *Acetobacter* culture in the presence of each dye and the cellulose regenerated from it were characterized with an X-ray diffractometer, solid-state ^{13}C nuclear magnetic resonance (^{13}C-NMR) and deuteration infrared (IR) spectrometry.

EXPERIMENTAL

Materials

Product Samples

2,2′-[4,4′-(3,3′-dimethyl)biphenylenebis(azo)]bis-[8-amino-1-hydroxy-naphthalene-3,6-disulfonic acid] tetrasodium salt (DB14; Aldrich Chemical Co.) and 2,2′-[4,4′-(3,3′-dimethyl)-biphenylenebis(azo)]bis[8-amino-1-hydroxy-naphthalene-5,7-disulfonic acid] tetrasodium salt (DB53; Aldrich Chemical Co.) were used as the probe dye. The method for the preparation of the samples was described in previous articles.[13] A cellulose-free cell suspension of 60 mL (*A. xylinum,* IFO 13693) was added to 140 mL of Hestrin–Schramm's medium (pH 7.0) containing a dye with concentrations of 0.1 wt %, and the mixture was incubated at 28°C for 24 h. The product was collected by centrifuging (6000 rpm, 5.15 g, and 20 min) at room temperature. To remove the excess dye, which is not related to dyeing of cellulose, the product from each DB14 and DB53 was rinsed only with distilled water for 1 h. These products were preserved at about 3°C in the wet state for the next experiments.

Regeneration of Cellulose from Product Sample

Nondried product samples were subjected to dye extraction by boiling in 70 vol % aqueous ethanol for 18 h (fresh aqueous ethanol solution was exchanged every 3 h). Residual samples were further boiled in a 1.0 wt % aqueous NaOH solution, neutralized with 1.0 vol % aqueous acetic acid, and subsequently rinsed with distilled water. This regenerated cellulose was preserved as above.

Standard Samples of Cellulose I and II

The cellulose I sample was obtained from microbial cellulose by boiling in a 1.0 wt % aqueous NaOH solution for 10 h under N_2 atmosphere, neutralized with 1.0 vol % aqueous acetic acid solution, and rinsed with distilled water. The cellulose II sample was prepared by mercerization of microbial cellulose at 20°C.

Methods

X-ray Measurements

In order to elucidate the structure of product, XRD of an uniplanar-oriented membrane of product sample was done by reflection and transmission. The uniplanar-oriented membrane was prepared on a Teflon plate and was dried at room temperature. On the other hand, diffractograms of isotropic samples of the product and cellulose regenerated from it were measured by transmission. In the latter case, the sample was compressed as a pellet to a 2 mm in diameter and 1-mm-thick pore of a sample holder. The X-ray diffractogram was measured by a MXP^{18} diffractometer (MAC Science) with Ni-filtered CuKα radiation. Measurement conditions were as follows: divergence slit, 1.0 mm; receiving slit, 0.15 mm; scanning speed, 4.0° min^{-1}; X-ray radiation, 40 kV, 200 mA.

Solid-State ^{13}C-NMR Measurements

Solid-state ^{13}C-NMR were performed at room temperature on a JEOL JNM EX 270 spectrome-

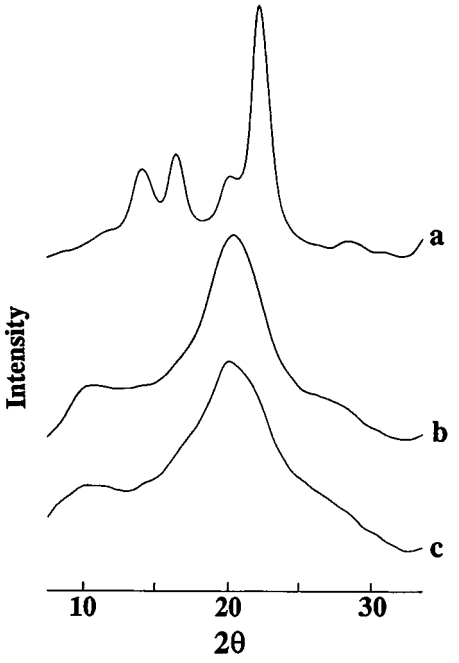


Figure 2 X-ray diffractograms of microbial cellulose and product samples from the *Acetobacter* culture in the presence of a dye of 0.1 wt %: (a) microbial cellulose; (b) DB14 product; (c) DB53 product.

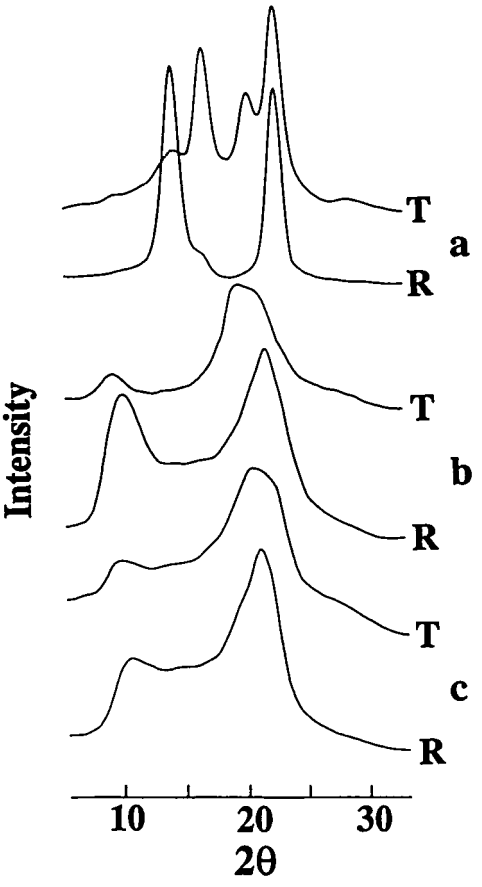


Figure 3 X-ray diffractograms of microbial cellulose and product samples measured by transmission (T) and reflection (R) methods: (a) microbial cellulose; (b) DB14 product; (c) DB53 product.

ter operating at 6.35 T, as described elsewhere.[3] The MAS rates were 5.0–5.5 kHz, and the chemical shifts relative to tetramethylsilane were determined by using the crystalline peak at 17.3 ppm of hexamethyl benzene as an external standard. Nondried samples were packed in a MAS rotor with an O-ring seal to avoid the loss of water during NMR measurements. Solid-state ^{13}C-NMR of crystalline component of regenerated cellulose was measured by Torchia sequence (τ : 50s).

Deuteration–Infrared Measurements

In order to obtain IR spectra of the sample, a membrane of about 10 μm in dry thickness was prepared from nondried samples and was dried on a Teflon plate. The deuteration method of the sample was performed by the method described in the previous article.[13] The membrane was set inside a glass cell so that the IR beam was perpendicular to the membrane surface. The glass cell that contained the sample was dried under reduced pressure (10^{-3} torr) for 2 h, and then the vapor phase deuteration of the sample was performed by D_2O (purity of 99.8%, Aldrich Chemical Co.) for a given time at room temperature. This was again dried under reduced pressure, and its

Table I X-ray Diffraction Angles of the Product Sample

Sample		2θ (deg)	2θ (deg)	2θ (deg)	2θ (deg)
MC	—	14.5 (w)	16.8 (w)	20.4 (w)	22.7 (s)
DB14	10.1 (vw)	—	—	20.6 (s)	—
DB53	10.3 (vw)	—	—	20.2 (s)	—

MC: microbial cellulose.

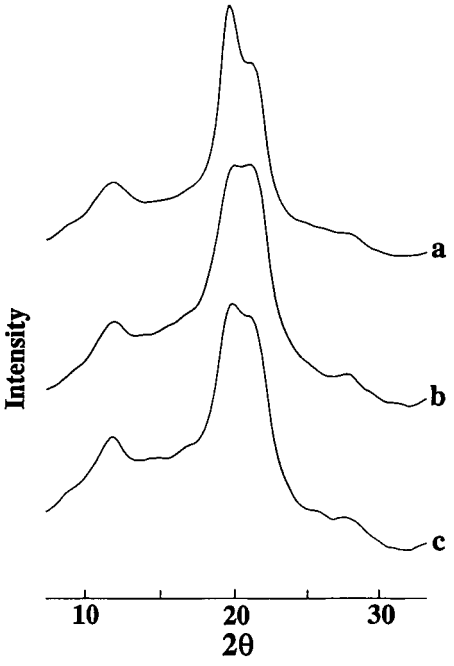


Figure 4 X-ray diffractograms of celluloses regenerated from product samples: (a) cellulose II; (b) DB14 cellulose; (c) DB53 cellulose.

IR spectrum was measured. A Fourier transform infrared (FTIR) system 800 (Nicolet) was used for the IR spectroscopy. The amount of OH and OD bands is calculated according to the method described.[13]

RESULTS

X-ray Diffractograms of the Product Sample

Figure 2 shows X-ray diffractograms of isotropic samples of microbial cellulose and DB14 and DB53 products. In Table I, 2θ values of diffraction peaks of each sample are listed. In Figure 3, X-ray diffractograms of uniplanar-oriented product samples measured by transmission and reflection are shown.

Table II X-ray Diffraction Angles of the Regenerated Cellulose Samples

	2θ (deg)		
Sample	$(1\bar{1}0)$	(110)	(020)
DB14	12.0	20.0	21.0
DB53	11.8	19.9	21.6
Cellulose II	12.0	19.8	21.1

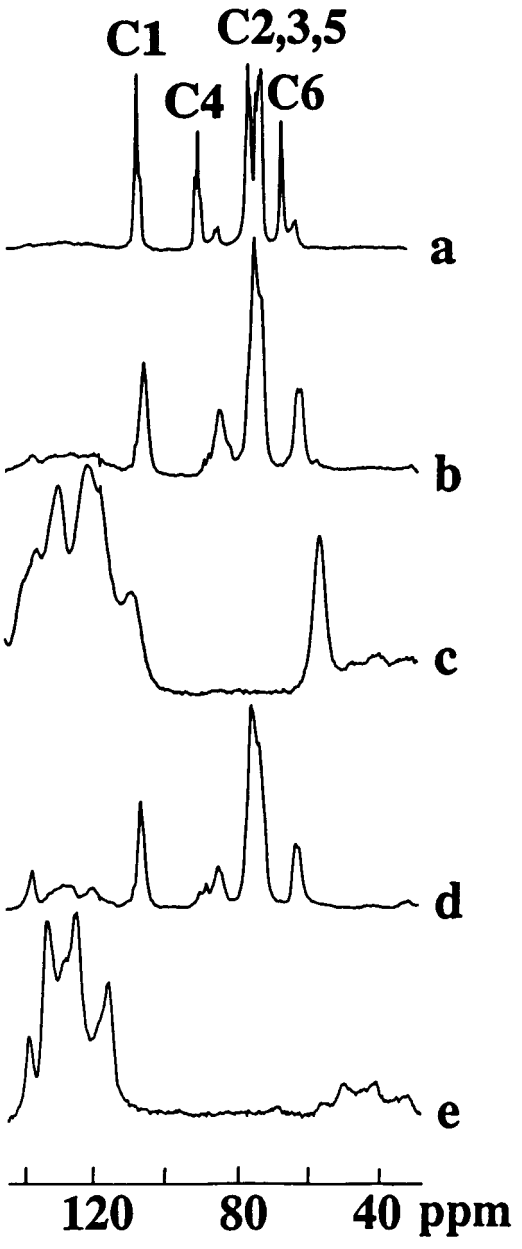


Figure 5 Solid-state ^{13}C-NMR spectra of the wet microbial cellulose, product samples, and dry direct dyes: (a) microbial cellulose; (b) DB14 product; (c) DB14 powder; (d) DB53 product; (e) DB53 powder.

As is apparent from Figure 2 and Table I, diffraction patterns of products are different from that of microbial cellulose. In the diffraction diagram of microbial cellulose, peaks of the $(1\bar{1}0)$, (110), and (020) planes occur at 2θ : 14.5, 16.8, and 22.7°. In contrast, these diffraction peaks of microbial cellulose disappeared in the products, but 2 new peaks appear, one of which occurs near 10°; the other is near 20°. These diffractograms

are almost the same to those of the products obtained from *Acetobacter* culture in the presence of DB1 and DB15 (DB1 and DB15 products), as reported in the previous article.[13]

The peak in diffraction diagrams of the uniplanar-oriented membrane of DB14 product at the lower angle side (2θ : 10°), obtained by reflection, is stronger than that obtained by transmission and is shown in the Figure 3(b). In contrast, the intensity of the diffraction peak at the higher angle side obtained by reflection is almost the same to that obtained by transmission. Similarly, the diffraction peak at the lower angle side obtained by reflection is somewhat stronger than that obtained by transmission in the diffractogram of DB53 product; whereas, in the case of transmission, the intensity of the diffraction peak at the higher angle side shows the same behavior as the DB14 product membrane. The uniplanar-orientation of these two products is similar to those of DB1 and DB15 products. Diffractograms of both product samples obtained by transmission are also almost the same as those of their isotropic samples means, and the product membranes do not attain uniplanar orientation like microbial cellulose or DR28 product.[13]

X-ray Diffractograms of Cellulose Regenerated from the Product Sample

Figure 4 shows X-ray diffractograms of the isotropic samples of cellulose regenerated from DB14 and DB15 products. Table II contains 2θ values of diffraction peaks of each sample.

As apparent from Figure 4 and Table II, celluloses regenerated from DB14 and DB53 products (DB14 and DB53 celluloses) show the typical diffractogram of cellulose II. This result is similar to that of cellulose regenerated from both DB1 and DB15 products.[13] The skeletal structure and substituent groups of DB14 and DB53 are the same as those of DB15 and DB1, except for the methyl substituents. The former contains methyl groups, while the latter contains methoxy groups. The coincidence of the crystal type of regenerated cellulose of the former and the latter suggests that the influence of methyl and methoxy groups on the structure of nascent microbial cellulose is negligible.

Solid-State ^{13}C-NMR Spectra of Product Sample

Solid-state ^{13}C-NMR spectra of microbial cellulose, DB14 and DB53 products, and their dye powders are shown in the Figure 5. In Table III, the chemical shifts of the resonance lines of the cellulose component in microbial cellulose and DB14 and DB53 products are listed.

It is clear from Figure 5 that no resonance lines of the cellulose component of DB14 and DB53 products overlap the lines of their corresponding DB14 and DB53 powders. It is therefore possible to discuss the structure of the cellulose component in product samples using ^{13}C-NMR spectra. From the Figure 5, it is clear that the ratio of dye to cellulose in the DB14 and DB53 products is low. The larger solubility of these dyes seems to remove not only the dye, which is not involved in the dyeing of the cellulose, but also the dye that has interacted with cellulose to form the product, during the rinsing process in the preparation of the sample. Due to the removal of some dye from the product, two small peaks are observed near C4: 89.5 and 88.5 ppm, respectively, which are the chemical shifts of crystalline component of conceived cellulose II in the product.

Furthermore, resonance lines of each carbon of cellulose component in the product are broader than those of microbial cellulose. Moreover, as apparent from Figure 5 and Table III, the chemical shifts of C4 and C6 resonance lines of each DB14 and DB53 product are at approximately 85 and 63 ppm, respectively, which are the chemical shifts of the noncrystalline cellulose component of microbial cellulose. This result suggests that, judging from the mobility of the carbons of the glucose residue, the cellulose component in the product is in a noncrystalline state regardless of dye included in the prod-

Table III ^{13}C-NMR Chemical Shifts (ppm) of Product Samples

Sample	C1	C4		C2	C3	C5	C6	C6
MC	106.0	89.9	84.7	75.5	73.4	72.1	66.2	62.5
DB14	105.2	—	85.0	75.2	—	—	—	62.2
DB53	105.6	—	85.0	75.5	—	—	—	62.9

MC: microbial cellulose.

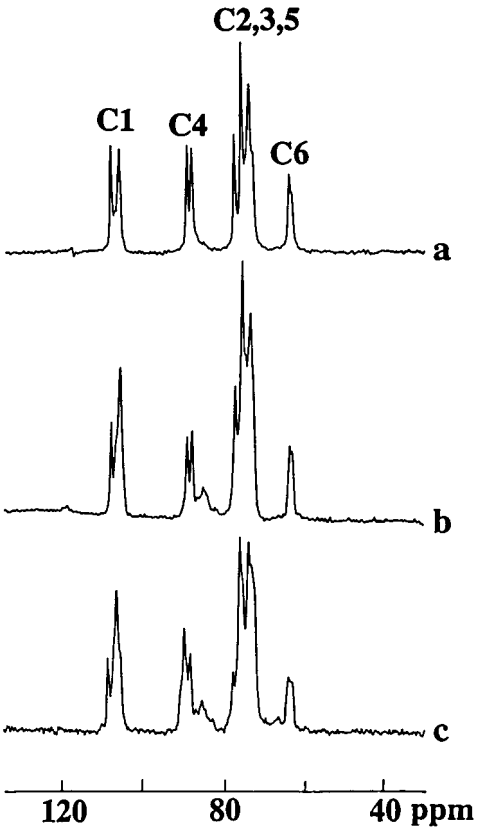


Figure 6 Solid-state ^{13}C-NMR spectra of crystalline components of the wet cellulose regenerated from products and cellulose II: (a) cellulose II; (b) DB14 cellulose; (c) DB53 cellulose.

uct. This ^{13}C-NMR result of product sample is inconsistent with X-ray measurement results, where the product is a crystalline complex. Both spectra of DB14 and DB53 products are similar to each other. These spectra also resemble very closely those of DB1 and DB15 products.[13]

Solid-State ^{13}C-NMR Spectra of Cellulose Regenerated from Product Sample

Figure 6 shows solid-state ^{13}C-NMR spectra of the crystalline component of cellulose regenerated from products and cellulose II obtained by mercerization of microbial cellulose. The chemical shifts of the resonance lines of regenerated celluloses and cellulose II are listed in Table IV.

^{13}C-NMR spectra and the chemical shifts of C1, C4, and C6 resonance lines of DB14 and DB53 celluloses indicate that these regenerated celluloses are cellulose II. The crystal structure of the regenerated celluloses are in good accord with their X-ray results. Although the DB14 and DB53 celluloses are cellulose II, the weak downfield peak of the Cellulose resonance line indicates that their fine crystal structure is somewhat different from that of cellulose II (mercerized microbial cellulose).

IR Spectra of Product Sample

The change in IR spectra of DB14 and DB53 products by vapor phase deuteration at room temperature is shown in Figure 7 and in Table V, the amounts of accessible OH group (OH group in that part of cellulose where OH groups can easily be transformed to OD group by the diffusion of D_2O) and nonaccessible OH groups (OH groups in that part of cellulose where the diffusion of D_2O is very difficult) in the product are listed.

The amount of nonaccessible OH groups in the DB14 product is 31%, and that in the DB53 product is 34%. After deuteration, the spectrum of the OH groups absorption of these samples indicates the structure of cellulose II. This indicates that some celluloses are regenerated from the product by the removal of dye during the rinsing process and that they crystallize to cellulose II; and, as a result, absorption bands that appears after deuteration are identical to cellulose II. Due to the inclusion of dye between cellulose sheets, the intra- and intermolecular hydrogen bonding between the cellulose sheets in the product is hindered. As there is no hydrogen bonding between cellulose sheets in the products, the amount of accessible OH groups in DB14 and DB53 products are 69 and 66%, respectively, much higher than for microbial cellulose (15%).

Table IV ^{13}C-NMR Chemical Shifts of the Crystalline Component of Regenerated Celluloses and Cellulose II

Sample	C1	C1	C4	C4	C4	C2	C3	C5	C6
DB14	107.9	105.5	89.5	88.2	85.3	77.3	75.6	73.4	63.6
DB53	108.3	106.2	90.1	88.7	85.6	77.8	75.9	73.8	64.2
Cellulose II	107.5	105.3	89.3	87.9	—	77.0	75.2	72.9	63.2

IR Spectra of Cellulose Regenerated from Products

Figure 8 shows the change in IR spectra of cellulose regenerated from the product by vapour phase deuteration at room temperature. The amounts of accessible OH (OD groups) and nonaccessible OH groups are listed in Table VI.

OH group absorption of DB14 and DB53 celluloses have two peaks at 3485 and 3449 cm^{-1} due to intramolecular hydrogen bonding, and one peak at 3340 cm^{-1} and one shoulder at 3179 cm^{-1} due to intermolecular hydrogen bonding.[15] These are the characteristic absorption bands of cellulose II. The absorption of OH groups by intermolecular hydrogen bonding at approximately 3340 cm^{-1} of DB14 cellulose is weaker than that of DB53 cellulose. This means that the intermolecular hydrogen bonding patterns between these two regenerated celluloses are not similar.

Table V Amount of OH and OD Bands of the Product and MC

Sample	OH Band (%)[a]	OD Band (%)
MC	85	15
DB14 product	31	69
DB53 product	34	66

MC: microbial cellulose.
[a] Nonaccessible OH band.

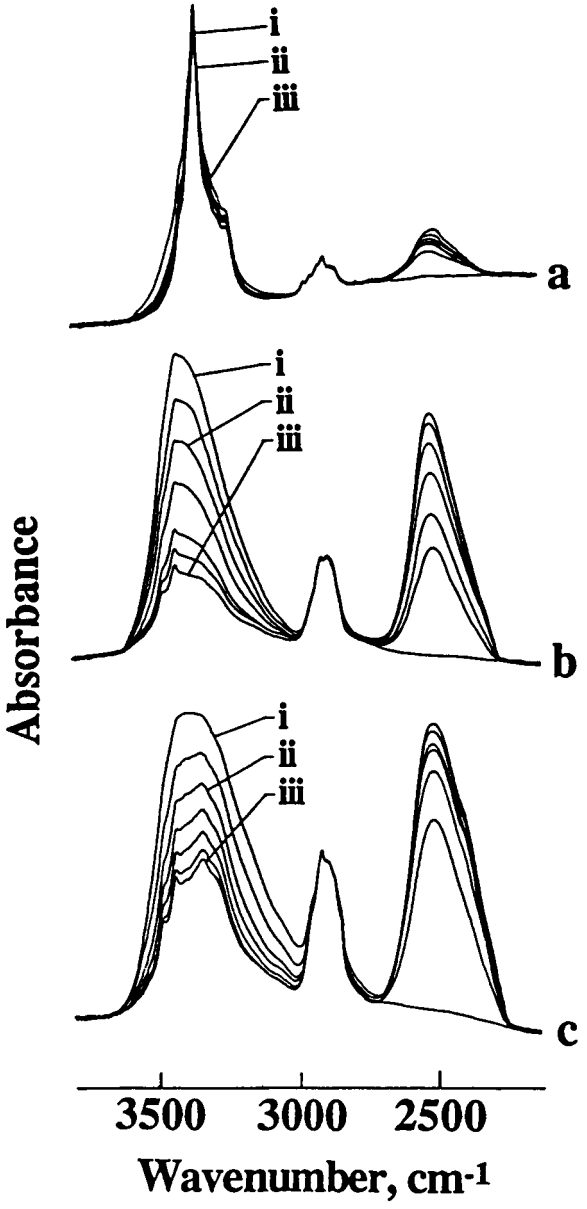


Figure 7 Change in IR spectra of product samples by vapor phase deuteration at room temperature: (a) microbial cellulose; (b) DB14 product; (c) DB53 product. Deuteration time (min); (i) 0; (ii) 3; (iii) 1000.

DISCUSSION

As the ratio of dye to cellulose in the product is poor, it is clear from the solid-state ^{13}C-NMR spectra that some dyes washed off from both DB14 and DB53 products by the rinsing process during the preparation of the sample. It is apparent from the deuteration IR measurement results that cellulose II is contained in the product sample, which is caused of removal of dye from the product sample. The uniplanar orientation of these product samples is similar to those of DB1 and DB15 product membranes.[13] Namely, X-ray diffractograms of membranes of products obtained in the presence of DB14 and DB15 having two sulfonate groups at 3 and 6 in the naphthalene nucleus resemble each other very much, and those of products obtained in the presence of DB1 and DB53 having two sulfonate groups at 5 and 7 in naphthalene nucleus also resemble each other very much. The dye having sulfonate groups at 3 and 6 of naphthalene nucleus reacted with nascent microbial cellulose; as a result, better orientation of the product in the diffraction plane occurs at lower angle side, and this plane is parallel to the surface of the membrane. This effect seems to occur in the IR spectrum of deuterated cellulose regenerated from their products. In spectra of the nonaccessible OH band of DB14 cellulose, the absorption of the intermolecular hydrogen bonding

near 3340 cm^{-1} is weak, and that in of DB53 cellulose is strong. Thus, due to the position of sulfonate groups in the naphthalene nucleus, a different uniplanar orientation of the product membrane as well as a different absorption of regenerated cellulose are observed. From these results, it is assumed that dye molecules may bind to cellulose chains through hydrogen bonding or van der Waals forces. From this discussion, it is clear that the position of sulfonate groups in the naphthalene nucleus in a direct dye affect the dye–cellulose interaction.

Although the washed off dye from the product causes fine crystals of cellulose II in DB14 and DB53 products, resonance lines of each carbon of the cellulose component are broad, and the chemical shifts of C4 and C6 correspond to that of noncrystalline component of microbial cellulose. It is suggested that in the complex component in the product (in the case of solid-state ^{13}C-NMR and deuteration IR spectroscopies), the dye is included between cellulose sheets corresponding to the $(1\bar{1}0)$ plane of microbial cellulose, that is, the (100) plane of the complex.[13] As the dye molecule is included between cellulose sheets and is formed in the noncrystalline product, dye molecules seem to affect the conformation of cellulose chains during regeneration. Due to this reason, cellulose II is regenerated from the product sample instead of I_α-rich cellulose of ordinary microbial cellulose.

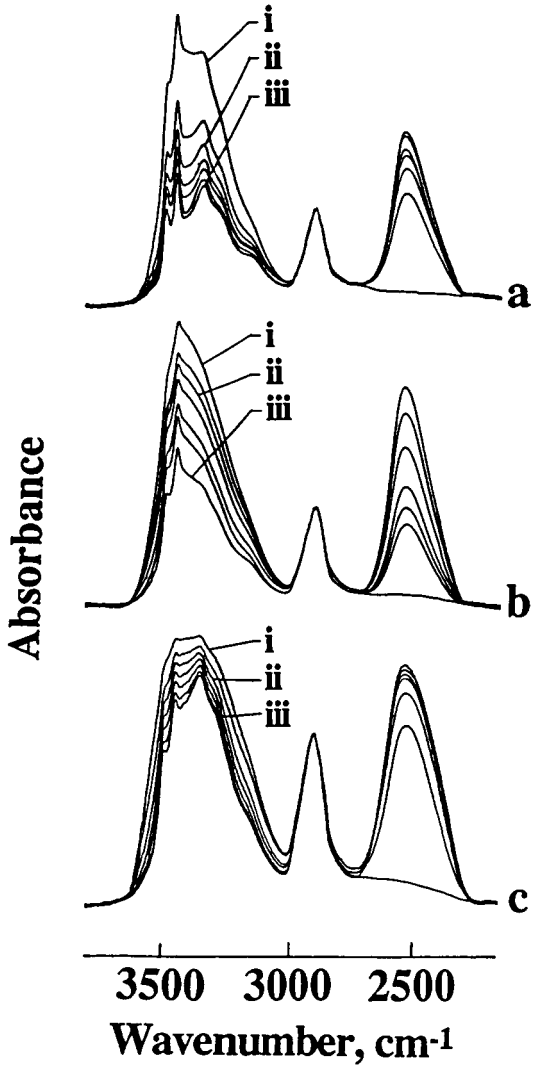


Figure 8 Change in IR spectra of celluloses regenerated from product samples by vapor phase deuteration at room temperature: (a) cellulose II; (b) DB14 cellulose; (c) DB53 cellulose. Deuteration time (min): (i) 0; (ii) 3; (iii) 1000.

Table VI Amount of OH and OD Bands of Regenerated Cellulose

Sample	*OH Band (%)	OD Band (%)
Cellulose II	45	55
DB14 cellulose	50	50
DB53 cellulose	48	52

[a] Nonaccessible OH band.

CONCLUSION

The influence of the location of sulfonate groups of DB14 and DB53 on the structure of the product, as well as the cellulose regenerated from it, does not differ from each other except for the influence of the dye on the uniplanar orientation of the product membrane and on the fine structure of regenerated cellulose. Substituent methyl and methoxy groups in the skeletal structure of dyes does not affect the structure of nascent microbial cellulose.

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