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Effects of reactive dyes on the enzymatic depolymerization of cellulose

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Abstract

Dyeings on cellulose produced with four different azo reactive dyes were exposed to the action of cellulase enzymes. The analysis of the resultant fabrics indicated that the level of depolymerization decreased with increasing concentration of covalently fixed dye. It was also found that the rate and extent of depolymerization also depended on the structure of the bonded dyes and that the dye concentration in the enzymatically liquefied fraction exceeded the level in the corresponding solid residue. The disparity between the fixed dye content associated with heterobifunctional reactive dyes and the ability of these dyes to decelerate cellulose depolymerization can be explained by the formation of dye–dye interactions between dye molecules fixed to cellulose and dye molecules undergoing exhaustion from the dyebath.

Keywords: Cellulases; Enzymatic hydrolysis; Reactive dyeing; Dye–fibre bonds; Dye-to-dye bonds

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1. Introduction

Extensive attention has been given to identifying treatments of cellulosic substrates that enhance their accessibility to enzymes. Such treatments can be classified as:

1. physical (beating, milling, irradiation) [1,2];
2. chemical (dissolution and reprecipitation, interaction with acids, bases and oxidising agents) [3–5];
3. thermochemical (thermohydrolysis, steam explosion, organosolv) [6–8]; and
4. biological (action of rot fungi) [9].

A possible application of the well controlled depolymerization of properly pretreated dissolving pulp cellulose is the ability to completely solubilize a cellulosic substrate in alkali. The dissolved "biotransformed" cellulose can be reprecipitated with acid in different forms (e.g. fibre, film) without serious environmental pollution [10].

Multiple mixtures of cellulase enzymes, produced by many different naturally occurring microorganisms effect the biodegradation of cellulosic

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substances, as an essential component of the carbon balance in the biosphere. The most important members of cellulose enzyme compositions are the endo-1,4-β-glucanases and exo-1,4-β-glucanases. The former types randomly attack cellulose molecules within the supermolecular structure of the substrate, and chain fragments of decreased molecular weight and new accessible end groups are formed as the result of this process. The resultant molecules are attacked by exo-1,4-β-glucanases at their non-reducing end and by cellobiosehydrolase. The products of the latter step are mixtures of oligosaccharides, cellobiose and glucose molecules, along with slightly depolymerized polymer chains. Cellulases, therefore, are very effective in the decomposition of cellulosic waste materials.

The influence of the derivatisation of cellulose on the rate and extent of its cellulase-induced degradation is a point of interest, as the generation of water soluble depolymerization products requires ready access of exo-glucanases and cellobiosehydrolase to the polymer chains. Their action, however, is affected by the location of substituted glucose residues along the chain backbone. For instance, sequential degradation by exoglucanases is blocked by the presence of substitutents on the repeating units. Consequently, monomer, dimer or oligomer units will be removed from a substituted cellulose chain only up to the point of substitution from the non-reducing end [11,12].

Dyes fixed to cellulosic substrates by primary and/or secondary forces behave similar to derivatising substituents during the enzymatic depolymerisation of dyed cellulosics. A drop in cellulose-induced weight loss has been reported for cotton dyed with direct [13,14] and reactive dyes [14,15]. In this regard, it was shown that the observed weight loss correlated with the morphological structure of the cotton and the fineness of the yarns [14–16].

Buschler-Diller [25] reported an initial slight increase in the disorder of cotton dyed with a direct dye during the course of enzymatic hydrolysis. However, no significant difference was found between the weight loss of the dyed and undyed fabrics. Dyeings derived from monofunctional or homobifunctional azo reactive dyes inhibit enzymatic hydrolysis to various degrees. While the type of the reactive group does not play an important role in a homobifunctional, a VS-VS type reactive dye proved to be the most efficient inhibitor, due to crosslinking of cellulose chains.

The goals of present study were to determine:

1. How dyeings from heterobifunctional azo reactive dyes influence the rate and extent of the enzymatic hydrolysis of the cellulosic substrate;
2. How the covalently fixed dye content is distributed between the liquid and solid phases after enzymatic hydrolysis;
3. How the covalently fixed dye content influences the rate and extent of enzymatic hydrolysis;
4. What types of dye-to-fibre and dye-to-dye bond interactions occur during dyeing with heterobifunctional azo reactive dyes;
5. Whether differences in% fixation and colour correlate with the amount of solid residues obtained after enzymatic hydrolysis.

The characterization of environmental problems caused by reactive dyes in the biodegradation of textile wastes was a further aim of this work.

2. Experimental

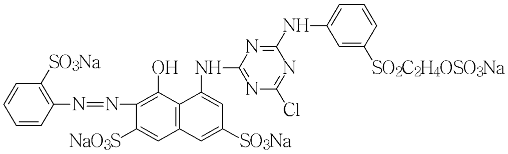
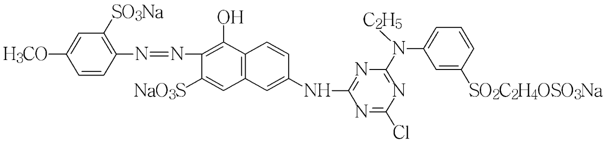
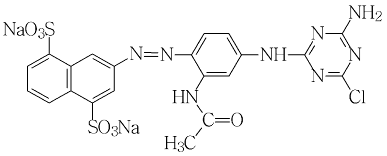
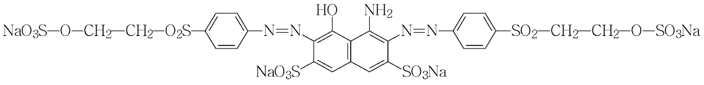
2.1. General

Plain woven desized, scoured, bleached and mercerised cotton fabric (108 g/m^2, $DP_V = 1495 \pm 50$) was used in this study. The dissolving pulp employed ($DP_v = 756 \pm 50$) was obtained from Fibrenier, USA.

2.2. Dye Application

The dyeing procedures were performed according to the manufacturer's technical information. Nominal dye bath concentrations (n.d.c.) in g dye per 100 g substrate were 0.1, 0.5, 1.0, 2.0, 4.0, 7.0, 10.0 and 15.0. The dyes used in this study are listed in Table 1 and were obtained from Sumitomo Chemical Company.

Table 1
Reactive dyes employed in this study

Code	Structure	Molar mass	Absorption max (nm)
A(MCT-VS)		983	518
B(MCT-VS)		938	502
RY-3(MCT) C.I. Reactive Yellow 3		637	380
RB-5(VS-VS) C.I. Reactive Black 5		1015	599

2.3. 3,4-β-Glucosidase activity (β-GA) [18,19]

p-Nitrophenyl-β-D-glucopyranoside (1 ml, 5 mmol in 0.05 M citrate buffer, pH = 4.8) and 0.1 ml enzyme solution were mixed in a test tube and after incubating at 50 °C for 10 min, Na_2CO_3 (2 ml, 1 M) and distilled water (10 ml) were added at room temperature. The absorbance values were measured photometrically, thereafter, at 400 nm, with enzyme activity calculated according to a previously reported method [18].

2.4. Reducing saccharide analysis [17]

The original (or diluted) liquid fraction from the enzyme-treated fabric (1.5 ml), the glucose content of which did not exceed 2 mg, was mixed with 3 ml of 3,5-dinitrosalicylic acid reagent. The yellow colour turned to brown upon heating the solution for 5 min in a boiling water bath. Changes in the absorbance of this solution, using 1.5 ml distilled water as a blank, were measured with an Ultraspec UV/visible spectrophotometer. A calibration curve, obtained with a set of glucose solutions of known concentrations, was used to determine the reducing saccharide content.

2.5. 3,5-Dinitrosalicylic acid reagent

3,5-Dinitrosalicylic acid (10 g) was dissolved in NaOH (500 ml, 0.5 M). Phenol (2 g), and Na_2SO_3 (0.5 g) were added, and after heating to 70 °C the potassium sodium salt of L(+) tartaric acid (200 g) was added. The solution was cooled to room temperature and the volume was raised to 1000 ml using distilled water.

2.6. Filter paper activity (FPA) [10]

Enzyme solution (0.1–1.0 ml), depending on its predetermined activity) was transferred to a test tube containing citrate buffer solution (1.5 ml). A piece of folded Whatman No. 1 filter paper (50 mg) was dipped into the enzyme solution and the resultant paper was incubated at 50 °C for 1 h. The reducing saccharide content was determined and FPA was calculated according to a previously reported method [17].

2.7. Biodegradation (% conversion)

Commercial cellulase enzymes (Table 2) were employed in this study. Celluclast and Pulpyzym are predominantly endo-glucanases, whereas Novozym functions mainly as exo-glucanases [20,21].

Cotton fabric (0.1 g) was dipped into a solution composed of Celluclast 2L (0.2 ml), Novozym 188 (0.2 ml) and citrate buffer (19.6 ml), and transferred to a sealed flask. The flask was incubated at 50 °C for 24 h, and aliquots were removed from the liquid after 1, 3, 5 and 24 h of incubation. The reducing saccharide (glucose) concentration in the liquid phase was measured and the% conversion was calculated using the following equation:

$$w = \frac{100\ c_{\mathrm{gl}} V}{1.1\ S}$$

$w = \%$ conversion; c_{gl} = glucose concentration (mg/ml) in the liquid phase; V = total volume (ml) of the liquid phase; S = dry weight of the substrate (mg); 1.1 = correction factor for the glucose molecules and their anhydroglucose units in the cellulose chain molecules $\left(\frac{180}{162} = 1.1\right)$.

The weight loss of the enzyme treated cotton was determined after the dwell time of 24 h.

2.8. Total dye content

Sulphuric acid (8 ml, 96%) was cooled to below 5 °C and finely ground dyed cotton fabric (0.05 g) was carefully added with stirring and extended cooling for 3 h. After complete dissolution the solution was very carefully diluted to 25 ml with distilled water [22]. Calibration curves for the dyes employed were used to determine the dye concentration in the cotton substrate. The soluble fraction was separated from the solid residue, prior to the dissolution of the latter in sulphuric acid, with the aid of a centrifuge (10 min at 10,000 rpm).

The UV/visible spectra of both liquid phases (enzyme solubilised and sulphuric acid soluble) were recorded using an HP 8452A diode array spectrophotometer.

2.9. Reflective colour measurement

Measurements made before and after enzyme treatment enabled the assessment of the colour differences.

3. Results and discussion

3.1. Most efficient composition of cellulase enzymes

Enzymes discussed in Table 2 depolymerize cellulosic substrates less efficiently following reactive dye fixation. The rate and level of enzyme catalyzed depolymerization of cellulosic substrates was too low when the enzymes listed in Table 2 were applied separately. When a 1:1 combination of the exo-glucanase and endo-glucanase enzymes was used, a significantly improved depolymerisation occurred. The most efficient depolymerization was achieved using 1:1 mixture of Celluclast 2L and Novozym (Table 3). Therefore, this system was used for the remainder of our experiments.

Table 2
Enzymes employed in this study

Trade name	Source	Filter paper activity (FPA) (Ee/ml)	β-Glucosidase activity (Ee/ml)	Producer
Celluclast 2L	*Trichoderma reesei*	49.08	18.86	Novo Industrias A/S (Denmark)
Novozym 188	*Aspergillus terreus*	3.37	199.78	Novo Industrias A/S (Denmark)
Pulpyzym		32.33	5.83	Novo Industrias A/S (Denmark)

3.2. Enzyme catalyzed hydrolysis of dyed pulp cellulose

The effects of bound reactive dyes on the enzyme-induced hydrolysis of dissolving pulp cellulose are shown in Tables 4 and 5. It can be seen that covalently fixed dye decreased the rate (g/100 g/h) of polymer chain hydrolysis. While both heterobifunctional reactive dyes caused a drop in the rate and level of hydrolysis, the actual effect depended on the structure of the dye. In case of the MCT-VS reactive dye A, the rate during the first hour decreased by 51% and by 71% after 24 h. The corresponding reduction by MCT-VS dye B was 58 and 96%, respectively.

Table 3
Conversion of dissolving pulp to reducing saccharides for enzyme catalysed depolymerisation

Enzyme	% Conversion[a]				% Weight loss[a]
	1 h	3 h	5 h	24 h	24 h
0.4 ml Celluclast 2L	13.99	32.59	39.70	58.87	60.50
0.2 ml Celluclast 2L + 0.2 ml Novozym 188	35.98	51.18	57.63	78.75	80.66
0.4 ml Pulpyzym	16.41	22.32	31.94	46.05	47.70
0.2 ml Pulpyzym + 0.2 ml Novozym 188	25.92	48.46	52.12	72.06	76.82

[a] After enzyme treatment.

Table 4
Changes in the kinetics and level of enzyme catalyzed hydrolysis of dissolving pulp cellulose caused by fixed reactive dye

Sample	Data	Duration of treatment (h)				Loss in weight in 24 h
		1	3	5	24	(%)
Undyed	Conversion[a]	35.98	51.18	57.63	78.73	85.58
	Rate of conv.[b]	35.98	17.06	11.52	3.28	3.57
Dyed with 7% A(MCT-VS)	Conversion	17.66	26.58	36.86	42.88	43.32
	Rate of conv.	17.66	8.86	7.37	1.79	1.81
Dyed with 7% B(MCT-VS)	Conversion	15.07	26.42	33.85	34.65	38.63
	Rate of conv.	15.07	8.81	6.77	1.44	1.61

[a] Conversion: g/100 g.

[b] Rate of conversion: g/100 g/h.

Table 5
Changes in conversion level and rate for the enzymatic hydrolysis of dissolving pulp during various time periods

Sample	Data	Period (h)			
		0–1	0–3	3–5	5–24
Undyed	Conversion	35.98	15.20	6.53	21.10
	Rate of conv.	35.98	7.60	3.26	1.12
Dyed with 7% A(MCT-VS)	Conversion	17.66	8.92	10.28	6.02
	Rate of conv.	17.66	4.46	5.14	0.31
Dyed with 7% B(MCT-VS)	Conversion	15.07	6.26	7.43	0.80
	Rate of conv.	15.07	3.13	3.71	0.04

Table 6
Changes in the kinetics of enzyme catalyzed hydrolysis of cotton cellulose caused by fixed reactive dye

Sample	Data	Duration of treatment (h)				Loss in weight in 24 h
		1	3	5	24	(%)
Undyed	Conversion[a]	13.18	21.95	27.24	56.21	69.83
	Rate of conv.[b]	13.18	7.32	5.45	2.34	
Dyed with 7% A(MCT-VS)	Conversion	8.98	12.05	16.36	31.10	33.89
	Rate of conv.	8.98	4.01	3.27	1.30	
Dyed with 7% B(MCT-VS)	Conversion	7.08	8.69	9.68	16.83	17.38
	Rate of conv.	7.08	2.90	1.94	0.70	

[a] Conversion: g/100 g.
[b] Rate of conversion: g/100 g/h.

3.3. Enzyme catalyzed hydrolysis of reactive dyed cotton fabric

The kinetics of enzyme catalyzed hydrolysis of dyed and undyed cotton fabric were followed for a 24 h period, the results of which are summarised in Tables 6 and 7. As would be anticipated, the hydrolysis of undyed cotton was faster than that of the undyed dissolving pulp cellulose. The presence of bound reactive dye decelerated and decreased the rate and level of hydrolysis markedly.

The rate of hydrolysis was highest during the early stages and the lowest after 24 h. In this regard, the decelerating effect of dye A (MCT-VS) was 32% after 1 h, and 48% after 24 h, while that of B(MCT-VS) was 62% after 1 h and 75% after 24 h. The inhibiting effect of fixed B(MCT-VS) dye on cotton exceeded that of fixed A(MCT-VS).

Table 7
Changes in conversion and rate of conversion for the enzymatic hydrolysis of cotton cellulose during various time periods

Sample	Data	Period (h)			
		0–1	0–3	3–5	5–24
Undyed	Conversion	13.18	8.77	5.29	28.97
	Rate of conv.	13.18	4.39	2.65	1.52
Dyed with 7% A(MCT-VS)	Conversion	8.98	3.07	4.31	14.74
	Rate of conv.	8.98	1.54	2.16	0.77
Dyed with 7% B(MCT-VS)	Conversion	7.08	1.61	0.99	7.15
	Rate of conv.	7.08	0.81	0.50	0.38

3.3.1. The action of unfixed reactive dyes

Experiments were conducted in order to determine whether the dyes themselves functioned as enzyme inhibitors or simply interfered with accessibility to the enzymes when covalently fixed to cellulose. In these experiments, hydrolysis of cotton cellulose was carried out using bicomponent (Celluclast 2L + Novozym 188) enzyme solutions containing 7% (owg) dissolved but unfixed A(MCT-VS) and B(MCT-VS). It was found that dissolved unfixed reactive dyes did not inhibit the action of the enzyme mixture (Table 8).

3.3.2. Enzyme catalyzed hydrolysis of cotton cellulose involving other reactive dyes

In this aspect of the study, the effects of heterobifunctional monoazo dyes A (MCT-VS) and B (MCT-VS) were compared to results involving the homobifunctional disazo dye RB-5(VS-VS) and the monofunctional monoazo dye RY-3(MCT). The effects of fixed dye content on the 24 h enzyme catalyzed hydrolysis are shown in Fig. 1

Table 8
Kinetics of enzyme catalyzed hydrolysis of cotton cellulose in the absence and presence of 7% unfixed reactive dye

Dye	Conversion (g/100 g) after			
	1 h	3 h	5 h	24 h
–	13.18	21.95	27.24	56.21
A(MCT-VS)	13.25	20.50	26.85	55.26
B(MCT-VS)	12.56	19.89	26.18	54.56

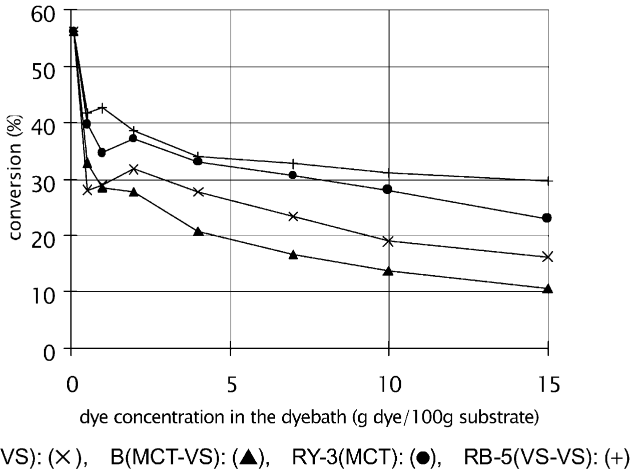


Fig. 1. Changes in the 24 h enzyme catalyzed hydrolysis of cotton cellulose caused by dyeing with reactive dyes.

and Table 9. In this case, the% cellulose conversion was plotted as a function of dye concentration in the starting dyebath. It was found that dye B(MCT-VS) caused the largest decrease in cellulose degradation. Here, the decrease was 50% and a minimum dyebath concentration of 2 g/100 g was needed. Dyebath concentrations of 4 g/100 g, 10 g/100 g, and >15 g/100 g were necessary to achieve the same results when A(MCT-VS), RY-3(MCT) and RB-5(VS-VS) were used. Dyeings involving a 0.1 g/100 g dyebath concentration caused a detectable decrease in hydrolysis, but differences among the four dyes were not significant.

The fixed dye content was determined spectroscopically after dissolving the dyed fabric in conc. H_2SO_4. The relationships between dye concentration and fixed dye content are summarised in Table 10. We found that a dyebath concentration of 1g/100g gave no significant difference in the fixed dye content among dyes A(MCT-VS), B(MCT-VS) and RY-3(MCT), whereas RB-5(VS-VS) gave much less fixed dye at this level. Increasing the nominal dyebath concentration up to 15 g dye/100 g led to the highest fixed dye content using B(MCT-VS). In this case, the amount of fixed dye was about twice the level obtained using A(MCT-VS) and RY-3(MCT). RB5(VS-VS) gave the lowest fixed dye content at all dyebath concentrations.

Changes in the 24-h enzyme catalyzed hydrolysis of cotton caused by fixed dye per 100 AGU are shown in Table 11. Initially, fixed dye content from the dyebath containing 1 g dye per 100 g substrate caused nearly identical (0.44–0.54%) "unit drops" in the polymer degradation following the application of A(MCT-VS), B(MCT-VS) and RY-3(MCT), with the drop caused by RB-5(VS-VS) twice as high (0.98%). The unit drop decreased to 0.07–0.20% for the two heterobifunctional dyes

Table 9
Changes in the 24 h enzyme catalyzed hydrolysis of cotton cellulose caused by fixed reactive dye

Dye	Drop in conversion (%) after 24 h, caused by x g dye/100 g substrate dyebath concentration					Dyebath concentration causing a 50% drop in conversion
	$x=0.1$	$x=1.0$	$x=4.0$	$x=7.0$	$x=15.0$	
RB-5(VS-VS)	23	25	37	43	46	$x>15$ g/100 g
RY-3(MCT)	29	37	41	46	59	$x=10$ g/100 g
A(MCT-VS)	37	48	50	54	71	$x=4$ g/100 g
B(MCT-VS)	37	48	64	71	80	$x=2$ g/100 g

Table 10
Fixed dye content [mmol dye/100 anhydroglucose unit (AGU)] following dyeing at various concentrations

Nominal dye concentration (g dye/100 g substrate in the dyebath)	Fixed dye content (mmole/100 AGU)			
	A(MCT-VS)	B(MCT-VS)	RY-3(MCT)	RB-5(VS-VS)
1.0	94.0	89.5	89.2	25.5
4.0	289.2	327.4	144.3	55.9
7.0	489.3	497.1	254.6	110.1
15.0	601.5	1126.1	561.6	296.9

Table 11
Changes in the hydrolytic conversion of cotton caused by various concentrations of fixed dye

Dye	Drop in conversion (%) caused by 1 mmol dye/100 AGU			
	Dyebath concentration (g/100 g)			
	1.0	4.0	7.0	15.0
A(MCT-VS)	0.51	0.17	0.12	0.12
B(MCT-VS)	0.54	0.20	0.14	0.07
RY-3(MCT)	0.44	0.28	0.18	0.11
RB-5(VS-VS)	0.98	0.67	0.39	0.16

were used and to 0.11–0.28 for the monofunctional dye, when the dyebath concentration was increased to 4–15 g/100 g. RB-5(VS-VS) gave a similar unit drop only at 15 g/100 g.

Since the decrease in the enzyme catalyzed hydrolysis of cellulose depends on the degree of substitution and the distribution of the substituents along the cellulose chain, specific points can be made concerning the results in Table 11. For instance, at a 1 g/100 g dyebath concentration, the initially high number of reactive sites on the cellulose chain makes dye–fibre bond formation occur readily. Increasing the dyebath concentration gives a higher ratio of dye molecules to a fixed number of reactive sites on the cellulose chain, suggesting that other pathways for dye fixation exist. A logical option would involve dye–dye bond formation [cf. Eqs. (1)–(3)]:

$$\begin{aligned}&\mathrm{Cell{-}OH + H_2C{=}CH{-}SO_2 - D{-}T{-}Cl} \rightarrow\\&\mathrm{Cell{-}O{-}CH_2{-}CH_2{-}SO_2{-}D{-}T{-}Cl}\end{aligned} \qquad (1)$$

$$\begin{aligned}&\mathrm{CH_2{=}CH{-}SO_2{-}D{-}T{-}Cl + H_2O} \rightarrow\\&\mathrm{HO{-}CH_2{-}CH_2{-}D{-}T{-}Cl}\end{aligned} \qquad (2)$$

$$\begin{aligned}&\mathrm{Cell{-}O{-}CH_2{-}CH_2{-}SO_2{-}D{-}T{-}Cl +}\\&\mathrm{HO{-}CH_2{-}CH_2{-}SO_2{-}D{-}T{-}Cl} \rightarrow\\&\mathrm{Cell{-}O{-}CH_2{-}CH_3{-}SO_2{-}D{-}T{-}O{-}}\\&\mathrm{CH_2{-}CH_2{-}SO_2{-}D{-}T{-}Cl}\end{aligned} \qquad (3)$$

As the VS groups hydrolysed earlier and faster than the MTC groups, dye-to-dye bonds might be formed with heterobifunctional dye molecules that were fixed to the fibre. This suggestion would explain the apparent disparity between results in Tables 9 and 10.

The experimentally obtained fixed dye contents (Table 10) were compared with the corresponding calculated values. Direct proportionality was assumed between the nominal dyebath concentration and the corresponding fixed dye content for the determination of the "calculated" values. The experimentally found fixed dye content at a dyebath concentration of 1 g/100 g was multiplied by 4, 7 and 15, respectively, in the calculations.

The results in Table 12 indicate that B(MCT-VS) gave the highest dye fixation efficiency at each dyebath concentration. While RB-5(VS-VS) gave the next best fixation efficiency, the actual fixed dye content was the lowest of the four dyes. Steadily declining dye fixation efficiency ($\frac{m}{c} \times 100$) was observed with increasing dyebath concentration when A(MCT-VS) was used, while after an initial large drop, fixation efficiency increased in the case of RY-3(MCT). It was demonstrated, recently [23,24], that dye–fibre bonds are due mainly to the VS-moiety in B(MCT-VS), whereas the MCT moiety bonds preferably when A(MCT-VS) is used. Only VS-type bonds can form from RB-5(VS-VS) and only MCT-type bonds from RY-3(MCT). At low dyebath concentrations, the fixation levels of A(MCT-VS), B(MCT-VS) and RY-3(MCT) were practically identical, while that of RB-5(VS-VS) was much lower. The formation of dye–dye bonds in dyeings involving the heterobifunctional azo reactive dyes A(MCT-VS and especially B(MCT-VS) is possible. This may involve MCT-type dye-fibre bond formation with simultaneous hydrolysis of the VS-group of the fixed dye molecule, to give a CH_2–CH_2–OH group onto which a dye molecule from the dyebath can be fixed. Another possible pathway is the formation of a VS-type dye–fibre bond without simultaneous hydrolysis of the MCT-group. In this case, dissolved dye molecules bearing a CH_2–CH_2–OH group could react with the MCT-group of the covalently fixed dye molecule.

No dye-to-dye bonds were formed that involve the monofunctional MCT dye, and none are likely in the case of the homobifunctional VS-VS type dye. For the latter dye, the kinetics of VS-fibre bond formation and that for hydrolysis of the second VS-group would need to be identical. At dyebath concentrations starting at 4g/100g, a steadily increasing proportion of the fixed B(MCT-VS) dye accumulated, without actually increasing the degree of substitution. In the case of RB-5(VS-VS), this type behavior occurred only near the dyebath concentration of 15 g/100 g.

3.4. The distribution of fixed dye between liquefied and solid cellulose

Enzyme degradation of cellulose gave a liquefied fraction, from dissolution of the amorphous regions, and a solid residue from the intact crystalline regions. The fixed dye contents in these two fractions were determined, and in this regard, colourimetric analysis employing calibration curves derived from reactive dye solutions was used to determine dye concentrations in the liquefied fractions. Subtracting the resultant values from the corresponding data in Table 10 gave the fixed dye content for the solid residue (Table 13). Based on the data in Table 13, it is clear that the higher the dyebath concentration the lower the proportion of fixed dye on cotton. The actual proportion depended on the reactive dye structure, with the highest level of fixed dye produced by B(MCT-VS), followed distantly by A(MCT-VS), RY-3(MCT), and RB-5(VS-VS). The fixed dye content in the liquefied fraction exceeded that of the untreated dyed cotton and the solid residue, unless the dyebath concentration exceeded 10 g/100 g.

Comparing the dye content in the liquefied fraction with that in the corresponding solid residue,

Table 12
Comparison of measured (M) and calculated (C) fixed dye contents at various dyebath concentrations

Nominal dye concentration (g/100 g)	A(MCT-VS)			B(MCT-VS)			RY-3(MCT)			RB-5(VS-VS)		
	M	C	$\frac{100M}{C}$ (%)	M	C	$\frac{100M}{C}$ (%)	M	C	$\frac{100M}{C}$ (%)	M	C	$\frac{100M}{C}$ (%)
1.0	94.0	94.0	100	89.5	89.5	100	89.2	89.2	100	25.5	25.5	100
4.0	289.2	376.0	76.9	327.4	358.0	91.5	144.3	356.8	40.4	55.9	102.0	53.2
7.0	389.3	658.0	55.2	497.1	626.5	80.0	254.6	624.4	40.8	110.1	177.5	62.0
15.0	601.5	1410.0	42.7	1126.1	1342.5	83.9	561.6	842.4	66.6	296.9	382.5	76.5

Table 13
Fixed dye contents in the initial (untreated) dyeings and enzymatically hydrolysed cotton fractions as a function of dyebath concentrations

Nominal dyebath concentration (g dye for 100 g substrate)	A(MCT-VS)			B(MCT-VS)			RY-3(MCT)			RB-5(VS-VS)		
	Dye content (mmole dye/100 AGU)			Dye content (mmole dye/100 AGU)			Dye content (mmole dye/100 AGU)			Dye content (mmole dye/100 AGU)		
	Initial	Liquefied	Solid	Initial	Liquefied	Solid	Initial	Liquefied	Solid	Initial	Liquefied	Solid
0.1	*	*	*	*	*	*	*	*	*	*	*	*
0.5	*	*	*	50.2	80.0	36.3	*	*	*	19.2	*	*
1.0	94.0	150.2	71.8	89.5	166.1	57.1	89.2	172.9	44.5	25.5	32.4	17.2
2.0	184.8	270.6	145.2	212.8	269.9	190.3	128.4	233.2	65.5	32.0	36.8	28.8
4.0	289.2	350.0	265.7	327.4	432.5	297.9	144.7	376.8	81.2	55.9	68.8	49.6
7.0	489.3	511.4	447.1	497.1	678.2	455.0	254.5	623.6	187.9	110.1	121.6	105.6
10.0	508.2	540.8	475.2	590.3	795.8	537.0	317.0	720.5	159.8	147.2	176.0	134.4
15.0	601.5	554.4	608.9	1126.1	1107.2	1124.5	561.6	1192.1	377. 1	290.9	280.0	294.2

Initial = dyebath.
* Less than 10 mmol/100 AGU.

the average ratio for the RY-3(MCT) dye was 4.2, while the other three dyes gave ratios between 1.2 and 1.9. Unlike the bireactive dyes, the monofunctional RY-3(MCT) dye cannot crosslink polymer chain segments in the amorphous region with chain segments on the surface of the adjacent crystalline region. Consequently, the amorphous regions retain high dye content following enzyme-induced separation from the crystalline regions. Since cellulase does not cleave dye–fibre bonds, bifunctional dyes are linked simultaneously to amorphous chain segments having high fixed dye content and the crystalline surface. The lower ratio of the fixed dye in the liquefied fractions to dye in the solid residues might be attributed to this phenomenon.

3.5. Reflective colorimetry

Reflective colorimetry was used to determine the colour differences between fixed dye in the untreated dyed cotton and the corresponding solid residues (Table 14). In these experiments it was found that treatment with citrate buffer only (no enzyme), did not remove dye or dyed fractions from the dyed fabrics.

The fixed dye content in the untreated dyed cotton significantly exceeded that in the solid residues when dyebath concentrations below 15 g/100 g were used. The lowest, but still significant colour difference was observed with B(MCT-VS) and the highest with RY-3(MCT).

The calculated $\frac{c_{\mathrm{i}} - c_{\mathrm{sr}}}{\Delta E^*_{\mathrm{ab}}}$ coefficient was practically constant in the dyebath concentration range of 1.0 through 10.0 g/100 g for the monofunctional dye RY-3(MCT). This indicated that a single dye–fibre bond was produced and that this process was independent of dye concentration. Similarly, but by an order of magnitude lower, a constant coefficient was obtained for RB-5(VS-VS) in the 1.0 to 7.0 g/100 g concentration range, and only slightly higher values were obtained at higher concentrations. While this would also be consistent with dye–fibre covalent bond formation, the simultaneous formation of dye–dye covalent bonds can be eliminated, apart from extremely high dye concentrations. In the absence of dye–dye bond formation, comparable differences between c_{i} and c_{sr} characterized the unit colour difference for A(MCT-VS), B(MCT-VS), and RY-3(MCT). Significantly lower $c_{\mathrm{i}}-c_{\mathrm{sr}}$ values caused identical colour differences, however, in the case if RB-5 (VS-VS). Critical dyebath concentrations can be detected in the production of dyeings from both heterobifunctional reactive dyes. A 2- to 3-fold

Table 14
Colour differences (ΔE^*_{ab}) between the initial (untreated) dyeings and solid residues obtained after enzymatic hydrolysis

Nominal dye concentration (g/100 g)	(ΔE^*_{ab})			
	A(MCT-VS)	B(MCT-VS)	RY-3(MCT)	RB-5(VS-VS)
0.1	10.6	6.1	5.1	8.3
0.5	8.0	6.4	9.4	7.3
1.0	7.7	6.9	13.0	8.5
2.0	8.7	7.3	13.3	7.7
4.0	6.6	4.2	14.1	8.5
7.0	6.0	4.1	12.0	9.3
10.0	4.8	5.9	11.2	8.8
15.0	–[a]	–[a]	7.4	–[a]

[a] The colour of the solid residue was darker than that of the initial dyeings.

Table 15
Differences in fixed dye content (mmol) between the initial (untreated) dyeings (c_i) and solid residues (c_{sr}) as a function of unit ($\Delta E^*_{ab} = 1$) colour differences reported in Tables 13 and 14

Nominal dye concentration (g/100 g)		Difference in fixed dye content (mmol)—$\left(\frac{c_i - c_{sr}}{\Delta E^*_{ab}}\right)$			
		A(MCT-VS)	B(MCT-VS)	RY-3(MCT)	RB-5(VS-VS)
1.0		2.88	4.70	3.44	0.98
2.0		4.55	3.08	4.73	0.42
4.0		3.56	7.02	4.50	0.74
7.0		7.03	10.0	5.55	0.50
10.0		6.88	8.98	5.11	1.45
	(first)[a]	(1.0–4.0)	(1.0–2.0)	(1.0–10.0)	(1.0–7.0)
Average		3.66	3.89	4.67	0.66
	(second)[b]	(7.0–10.0)	(4.0–10.0)	–	(10.0)

[a] The average for the $\left(\frac{c_i - c_{sr}}{\Delta E^*_{ab}}\right)$ coefficients was calculated in the range of nominal dye concentrations, where the time for dye–dye bond or aggregate formation has been excluded. (Average below the critical dye concentration.)

[b] The average for the $\left(\frac{c_i - c_{sr}}{\Delta E^*_{ab}}\right)$ coefficients was calculated in the range of nominal dye concentrations, where the time for dye–dye bond or aggregate formation has been included. (Average over a critical dye concentration.)

higher $c_i - c_{sr}$ difference is needed over the critical concentration for a unit difference in colour, as might be anticipated. Dye molecules that are directly fixed to cellulose hydroxyl groups contribute more efficiently to the colour of the dyed fabrics than those fixed by dye–dye bridging to cellulose. In this regard, B(MCT-VS) began bond formation at lower concentrations (2–4 g/100 g) than A(MCT-VS) (4–7 g/100 g) (Table 15). This is consistent with the previously mentioned differences between high fixed dye contents involving dyeings from A(MCT-VS) and B(MCT-VS) and the decrease in rate of enzyme catalysed hydrolysis of these dyeings.

4. Conclusions

An optimised mixture of two commercial cellulose enzyme preparations (Celluclast 2L and Novozym) effected reproducible depolymerization of native cellulose substrates. In this regard, covalently bonded reactive dyes reduced the rate and degree of depolymerization.

The dependence of the depolymerisation process on fixed dye content varied with the functionality of the reactive dye employed. Since monofunctional dye molecules could only be fixed to the accessible sites of cellulose, they have much less chance for fixation at the surface of the highly ordered regions than in the amorphous regions. The bifunctional dyes—especially the MCT-VS types at higher fixed dye contents—may form crosslinks within the highly accessible regions as well as between chain molecules of differently ordered domains.

Crosslinking within the substrate may strongly reduce enzyme accessibility. With this in mind, the correlation between the fixed dye content of the substrate and the decrease in depolymerisation makes the formation of covalent dye–dye bonds likely in the dyeings involving heterobifunctional reactive dyes, primarily at higher fixed dye contents. About eight RY-3(MCT) molecules are fixed to a cellulose chain of DP = 1500, in dyed cotton obtained from the dyebath of 15 g dye per 100 g substrate. At least 16 dye molecules are fixed under identical conditions when B(MCT-VS) is used. Either crosslinks or the formation of bimolecular dye–dye aggregates could account for the observed reduction in depolymerisation, which was less than expected.

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