Nonionic surfactants and dispersants for biopolishing and stonewashing with *Hypocrea jecorina* cellulases

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Received: 13 October 2011; Accepted: 22 May 2012

Cellulases are widely applied in textile finishing, such as for the removal of protruding surface fibrils to reduce pilling propensity and to achieve the worn-out look in denim garments. The main drawback of enzymatic denim processing is the back-staining of indigo, which reduces the desired blue–white contrast. Alongside an accurate selection of the type of cellulase or vigorous post-washing of the garments, the simultaneous application of auxiliaries in the enzymatic treatment may help to reduce back-staining and improve cellulase efficiency. In the present work, the influence of additives such as surfactants and dispersing agents on indigo adsorption and on the treatment of an undyed cotton fabric with *Hypocrea jecorina* cellulases was investigated. Indigo adsorption was successfully reduced by more than 75% with ethoxylated nonionic surfactants at concentrations below 0.2 g l⁻¹. The weight loss of cotton fabrics after 120 min treatment was significantly increased with nonionic surfactants and polyvinylpyrrolidone. It could be further shown that protein adsorption on the cotton fabric decreased with the increasing concentration of the additives, while the nonionic surfactants were more efficient than the polyvinylpyrrolidone. Adsorption of a complete cellulase mixture was affected differently by the surfactants than by an exoglucanase-free endoglucanase-rich preparation.

Introduction

Cellulases are widely used in the textile industry for the finishing of fabrics made from cellulosic fibres, and they play an important role in the hydrolysis of lignocellulosic biomass for the production of second-generation bioethanol [1]. Their main applications in textile finishing are: the removal of fuzzy surface fibres and microfibrils to create a smoother and brighter fabric surface with reduced pilling propensity (biopolishing); the removal of non-uniform first fibrillation of lyocell fibres (defibrillation of lyocell fibres); and the non-homogenous removal of indigo dye from denim garments to obtain an old and used characteristic (biostoning). Because of technical and environmental problems, the abrasive pumice stones previously used have been gradually substituted by cellulases in the washing of jeanswear. Beside the desired worn-out look, the enzyme treatment makes garments more flexible and comfortable and improves the fabric handling [2–6]. A drawback of the use of cellulases is indigo back-staining – the redeposition or readsorption of the dye onto the white threads (weft) of the denim fabric that reduces the blue/white contrast. This undesired effect is attributable to interactions of indigo with cotton cellulose and proteins from the cellulase preparation, it depends on the cellulase type and is especially observed with cellulases from *Hypocrea jecorina* (formerly *Trichoderma reesei*) [2,7,8]. Preferential binding sites for indigo on cellulase proteins have been identified [9,10].

Post-washing of cellulase-treated denim garments has been suggested to reduce the undesired back-staining effect, and detergents, surfactants and dispersing agents have been shown to remove indigo from stained cotton fabrics [11];

however, an additional step means longer process times and higher water consumption. The simultaneous application of these chemicals together with cellulases might be advantageous over the two-step procedure, as they might avoid dye redeposition on the fabric and have an additional positive effect on enzyme action.

Different chemicals, especially proteins and surfactants, have been reported to influence the hydrolysis of cellulosic and lignocellulosic materials with cellulases [6,12,13]. Cellulose hydrolysis may be divided into four fundamental steps, namely: (i) enzyme diffusion to the substrate, (ii) enzyme adsorption onto the substrate, (iii) hydrolysis of the β -1,4-glycosidic bond and (iv) desorption of the enzyme and soluble product from the insoluble substrate. Inefficient or non-productive adsorption of enzymes to lignin, non-cellulosic material and reaction vessel walls may be quite high (24% of cellulase and 42% of β -glucosidase protein have been reported to be lost because of unspecific adsorption on glass vessel surfaces), reduces cellulose hydrolysis and increases the demand for higher enzyme loads [13]. Non-catalytic proteins such as bovine serum albumin (BSA) can prevent cellulase adsorption onto glass [14] without affecting cellulase adsorption on cellulose [15] and seem to reduce unproductive adsorption on hydrophobic reaction vessel walls [13] and lignin [16], thus increasing the amount of available enzyme.

Surfactants and other auxiliaries together with cellulases have been reported to enhance their hydrolytic action [17–20] and diminish denaturation of enzymes subjected to shear forces such as in agitated systems, which are usually applied in the processing of textiles [17,21]. Surfactants and polymers were found to have a protective effect at much

lower concentrations ($0.01\text{--}0.1\text{ mg ml}^{-1}$) than proteins [21]. Interactions of the surfactant with the hydrophobic part of the protein may minimise aggregation and denaturation.

Different studies have shown that nonionic surfactants especially have a beneficial effect on the hydrolysis of cellulosic and lignocellulosic substrates, whereas anionic and cationic surfactants interfered negatively [17–19,22–26]. With nonionic surfactants increased formation of reducing soluble sugars and substrate conversion were reported. The effect depends on the substrate and was not observed for soluble substrates, such as carboxymethylcellulose or cellobiose [17].

Surfactants are more effective at lower enzyme loads and reduce the amount of adsorbed protein [17,22–24], which can be used to increase desorption of cellulase from the cellulosic substrate [27]. However, the use of surfactants to enhance desorption of cellulases from textile substrates in order to recover and recycle cellulases was not successful [28].

One possible explanation for the effect of surfactants on cellulose hydrolysis is that surfactants adsorb to the cellulosic substrate, lower the surface tension, improve the wettability of the substrate and make it more accessible for the enzymes [17]. Surfactants influence the adsorption process on the substrate, reducing the immobilisation of the enzyme on the cellulosic substrate and affect the degree of adsorption. In particular, nonionic surfactants such as Tween 20 were found to reduce the adsorption of endoglucanase activity to insoluble cellulosic substrates and enrich their concentration in the liquid phase, thus regulating the adsorption profile of exo- and endoglucanases on the cellulose surface [18,24]. For the same surfactant, different interactions with different enzymes must be expected.

The polyoxyethylene glycol chains of nonionic surfactants, expressed by the degree of ethoxylation (EO), were proposed to have two effects: (i) the occupation of the hydrophobic substrate sites and (ii) to reduce protein adsorption because of their volume [23]. It was further suggested that surfactants, when adsorbed to crystalline cellulose, might help to disrupt the hydrogen bonding environment and to avoid reannealing of the glycosidic bond after hydrolysis [17].

Although anionic surfactants can establish electrostatic interactions with positively charged amino groups of the protein, and denature and inactivate cellulases [25,26], this effect seems to be reversible when the enzyme is separated from the surfactant [28]. Eriksson *et al.* have also shown that the negative charge of surfactants such as sodium dodecyl sulphate (SDS) can amplify the effect of nonionic surfactants, preventing unproductive binding of negatively charged enzymes (isoelectric point of Cel 7A 3.9; at pH 4.8) to lignin (negatively charged due to surfactant adsorption). In mixed micelles with nonionic surfactants, sodium dodecyl sulphate is completely incorporated and the denaturing effect is reduced [23].

For cationic surfactants, both negative effects and enhancement of the hydrolytic action of cellulases were reported [25,26].

In the present work, the influence of surfactants and polymeric dispersants on the adsorption of indigo on a white cotton fabric and the influence of three of these additives on the treatment of an undyed cotton fabric with different preparations of *H. jecorina* cellulose was investigated.

Experimental

Indigo adsorption on cotton fabrics in the presence of additives

Swatches (2.45 g) of the plain woven cotton fabric, were incubated for 30 min at $50\text{ }^{\circ}\text{C}$ in 500 ml stainless steel recipients on a Washtester (Kimak, Brazil) at 40 rpm with 0.124 g pure macerated indigo (BASF, Germany), in 50 ml acetate buffer pH 5 (0.1 mol l^{-1} ; sodium hydroxide/acetic acid) and with different additives at varying concentrations (0, 0.01, 0.02, 0.05, 0.1, 0.2, 1.0 and 2.0 g l^{-1}). The nonionic-surfactants Lutensol AP10 (alkylphenol 10EO), Lutensol AP20 (alkylphenol 20EO), Lutensol AT25 (C16-C18 fat alcohol, 7EO), Lutensol ON70 (C10 oxoalcohol 7EO) and the dispersing agents Sokalan HP50 (polyvinylpyrrolidone; PVP) and Sokalan CP10 (modified polyacrylic acid) used as additives were kindly provided by BASF.

Indigo adsorption on the fabrics was evaluated by reflectance measurements using an Optronik (Germany) spectrophotometer and were expressed as the *K/S* value at 660 nm .

All experiments were carried out with a plain woven cotton fabric, that was prewashed in a domestic washing machine (Continental Evolution, program 4; Continental, Brazil) with $1\text{ g l}^{-1}\text{ Na}_2\text{CO}_3$ for 60 minutes. After neutralising in diluted acetic acid, the fabric was thoroughly rinsed with distilled water and dried on air.

Enzymatic treatments

Pieces of the prewashed cotton fabric (2.45 g) (Continental, programme 4), were treated in 50 ml acetate buffer at pH 5 (acetate buffer: 0.1 mol l^{-1} acetic acid/sodium hydroxide) with $5\text{ mg protein g}^{-1}$ fabric of *H. jecorina* cellulases over 120 min at $50 \pm 2\text{ }^{\circ}\text{C}$. Experiments were carried out in 500 ml stainless steel recipients on a Kimak Washtester with vertical agitation (40 rpm). Total crude (TC) cellulase, a complete cellulase mixture with the main components of: endoglucanases I and II (EGI and EGII) and cellobiohydrolases I and II (CBHI and CBHII); ($100\text{ mg protein ml}^{-1}$ determined by the Lowry method, activity: $16\text{ }000\text{ ECU ml}^{-1}$ e 65 FPU ml^{-1}), endoglucanase-rich, (with deleted CBH genes, --cbh1 and --cbh2 , and main components EGI and EGII in a protein ratio of 50/50) and CBH-rich (with deleted EG genes --eg1 and --eg2 , and main components CBHI and CBHII in a protein ratio of 85/15) were produced by genetically modified strains of *H. jecorina* and were kindly provided by AB Enzymes (Finland, formerly Roehm Enzymes). Cellulases EGI-enriched [1651 RBB-CMC (Remazol Brilliant Blue-carboxymethylcellulose) units g^{-1} sample] and EGII-enriched (10676 CMC units g^{-1} sample), without exo- β -1,4-glucanase activities (CBHI and CBHII) were kindly provided by Danisco (USA, formerly Genencor International). All enzymes were used without further purification.

Treatments were carried out with varying concentrations (0, 0.05, 0.2 and 2.0 g l^{-1}) of additives C10 oxoalcohol 7EO, C16-C18 fat alcohol 7EO and polyvinylpyrrolidone.

Fabric and treatment bath analysis

After the enzymatic treatments, fabrics were washed with 1% sodium carbonate at $60\text{--}80\text{ }^{\circ}\text{C}$ (to terminate the enzy-

matic action), neutralised with 1% acetic acid and finally washed with distilled water. Fabrics were air-dried and conditioned at 21 °C and 65% relative humidity over at least 24 h before weighing. Weight after the treatment was further corrected according to the weight variation observed for control fabrics that were weighed together with the treated fabrics without being submitted to any treatment. Weight loss was calculated as follows:

$$\text{weight loss(\%)} = (M_1 - M_2)/M_1 \times 100$$

where M_1 is the initial weight and M_2 is the weight after treatment.

Soluble reducing sugars in the treatment bath and insoluble reducing sugars on the fabrics were analysed using the neocuproine method [3,29,30]. Therefore a solution of 0.2 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (Vetec, Brazil) and 0.4 g neocuproine hydrochloride (Fluka, Switzerland) or the equivalent amount of neocuproine in 1 l of distilled water was prepared. Aliquots (50 μl) of the treatment bath were taken after 0, 30, 60 and 120 min and reacted with 5 ml neocuproine solution and 2 ml sodium carbonate (20 g l^{-1} ; Vetec, Brazil), boiled for 10 min and diluted with 10 ml distilled water. Absorption was read at 456 nm on a Shimadzu (Japan) UV-1601 spectrophotometer. For analysis of the insoluble reducing sugars on the fabrics, 150 mg fabric was added to 50 μl distilled water and reacted with neocuproine and sodium carbonate as described above. Reducing sugars were expressed as glucose equivalents, using glucose (p.a., Vetec) as a standard.

Soluble protein was determined according to the method described by Bradford [31]. No significant influence of the additives on reducing sugar and protein measurement were observed.

Results and Discussion

Indigo adsorption on cotton fabric in the presence of additives

Indigo back-staining or indigo adsorption onto cotton fabrics during enzymatic finishing of denim garments with acid cellulases is a well-known problem and has been previously studied in order to understand its mechanism and to diminish its undesired effect [7,8,11]. Alongside the selection of cellulases that cause less back-staining, post-washing with surfactants, dispersants or complex detergents was indicated as an efficient method (more than 80% colour removal) to reduce back-staining. The best results for one-component washings were reported for nonionic surfactants with a low degree of ethoxylation and a polyacrylate [11]. In the present study, the anti-redeposition effect of different nonionic surfactants and dispersing polymers was investigated by washing a cotton fabric sample together with indigo dye and different concentrations of the additives.

For all additives, except the polyacrylate, indigo dye adsorption onto cotton was drastically reduced for concentrations up to 0.2 g l^{-1} . Higher additive concentrations did not significantly improve the anti-redeposition effect (Fischer test, 95% confidence interval) (Figure 1). The lowest indigo adsorption was obtained with two nonionic alkylphenol surfactants and a long chain (C16–C18) fat

alcohol surfactant. In general, the nonionic surfactants proved to be more efficient at lower concentrations than the investigated dispersing agents. This can probably be attributed to the different dispersing mechanism of surfactants and polymeric dispersants and the fact that nonionic surfactants are able to form micelles at very low concentrations [9,10].

Influence of additives on enzyme treatment of cotton fabric

Three of the additives evaluated as anti-redeposition agents for indigo, namely ethoxylated (7EO) C10-oxo-alcohol and C16–C18 fat alcohol surfactants and polyvinylpyrrolidone, were further investigated with respect to their influence on cellulase action on undyed cotton fabrics. After 120 min treatment with the complete cellulase, a total crude significant increase in weight loss was observed for all three additives. For the nonionic surfactants, the highest weight loss was

Figure 1 Adsorption of indigo dye (in the oxidised form) onto a plain woven cotton fabric in the presence of additives in different concentrations

Figure 2 Weight loss after 120 min total crude cellulase treatment of plain woven cotton fabric at a 1:20 liquor ratio with different additives at varying concentrations (0–2 g l^{-1}). Values represent the difference between treatments without and with cellulase using the same additive concentration

already achieved at the lowest tested additive concentration (0.05 g l^{-1}), whereas an increase in polyvinylpyrrolidone concentration from 0.05 to 2.0 g l^{-1} seems to increase weight loss slightly. However, although weight losses were significantly higher in the presence of additives, essentially no statistical difference between weight losses for different additive concentrations and between different additives was observed (Figure 2).

Cellulase treatment of cotton fabrics causes weight loss, which is attributable to the liberation of reducing soluble sugars, such as cellobiose, glucose and smaller oligomers, and to the break-off of cellulose fibrils. Weight loss depends on several variables, such as cellulase type, treatment time, applied shear force and physical-chemical properties of the substrate. With the same enzyme load, the highest weight loss is expected for complete cellulases, because of the synergy between the different enzymes [32].

The effect of the nonionic surfactant C10 oxoalcohol was also investigated for two monocomponent cellulase preparations, EGI-enriched and EGII-enriched. For both

Figure 3 Weight loss of cotton fabrics as a result of 120 min treatment with cellulases EGI-enriched and EGII-enriched ($5 \text{ mg protein g}^{-1}$ fabric) without and with the addition of the nonionic surfactant C10 oxoalcohol 7EO at 50°C in a Kimak Washtester at 40 rpm vertical agitation

Figure 4 Reducing sugar production during a 120 min treatment of a plain woven cotton fabric with cellulase TC (or: Total Crude) at 1:20 liquor ratio and different concentrations of additives C10 oxoalcohol 7EO (left) and PVP (right) (0 – 2 g l^{-1})

cellulase preparations, a significant weight loss increase with the addition of the nonionic surfactant was observed, but no statistical difference between surfactant concentrations was found. From the comparison of Figures 2 and 3 it can be seen that the nonionic surfactant had a higher impact on the endoglucanases (EGI and EGII) than on whole cellulase preparations that contained exoglucanases (CBHI and CBHII). This result is in agreement with that reported by Ooshima *et al.* [24] and Park *et al.* [18], who commented that surfactants mainly affect endoglucanase adsorption to the substrate.

One possible explanation is that additives such as surfactants and dispersing agents facilitate the removal of reaction products from the fabric surface, protein desorption from the fibre surface and readsorption of cellulase enzymes, thus accelerating the hydrolytic turnover of the cellulosic substrate.

The three additives did not show any significant influence on the reducing groups on the surface of the cotton fabric after 120 min (results not shown). Considering previous studies [33], this might indicate that additives did not have any influence on endoglucanase enzymes; however, the reducing surface groups were only analysed after 120 min of treatment, and insoluble reducing sugars are removed by exoglucanase action.

The amount of soluble reducing sugars released during hydrolysis was slightly increased in the presence of the additives C10 oxoalcohol 7EO and polyvinylpyrrolidone; however, the change was less significant when compared with the variable of time and no significant difference between additives could be detected under the experimental conditions (Figure 4). Mizutani *et al.* [19] found similar results for cellulase action in the presence of the nonionic surfactant Tween 20 on avicel, lyocell and cotton yarns. They reported further that long incubation times (at least 8 h) were necessary to see the effect of the surfactant; however, according to the description of the experimental procedure, they used no or little mechanical shaking, whereas in these experiments rigorous vertical shaking was used. Ooshima *et al.* [24] reported that, between different nonionic surfactants, Tween 20 was most effective, attaining after 72 h a 35% saccharification increase for avicel (5 wt%) in the presence of 0.05 wt% Tween 20.

Results concerning the increased weight loss and the increase in reducing soluble sugars are also in agreement with former studies by Helle *et al.* [17], who found that the addition of nonionic biosurfactants and Tween 80 increased the initial rate of hydrolysis of Sigmacell 100; however, surfactant addition later in the process turned out to be less effective.

The increase in reducing soluble sugars may be explained by the synergism between different cellulase components [32]. As endoglucanase enzymes create new reducing groups on the fabric surface, these groups serve as new attack points for cellobiohydrolases (CBH), which in turn increase the formation of reducing soluble sugars.

Influence of additives on protein adsorption

Protein adsorption onto the cellulosic substrate is an essential step for cellulose hydrolysis; however, non-productive adsorption to reaction vessel walls and non-cellulosic materials might occur [13]. In this study, no distinction between unproductive protein adsorption and cellulose adsorption was made and, although some non-productive adsorption onto the reaction vessel might have occurred, results are discussed with respect to the adsorption of proteins onto the cotton fabrics. The amount of

Figure 5 Protein adsorption of total crude cellulase to a plain woven cotton fabric after 120 min of incubation (vertical agitation, 40 rpm; 1:20 liquor ratio; pH 5.0; 50 °C) in the presence of three different additives with increasing concentrations

Figure 6 Protein adsorption of total crude and EG-rich cellulase preparations to a plain woven cotton fabric after 120 min of incubation (vertical agitation, 40 rpm; 1:20 liquor ratio; pH 5.0; 50 °C) in the presence of C10 oxoalcohol 7EO with increasing concentrations

adsorbed protein after 120 min treatment with *H. jecorina* cellulases under conditions of vertical shaking and 50 °C was significantly decreased by the addition of at least 0.05 g l⁻¹ of the two nonionic surfactants, C10 oxoalcohol 7EO and C16-C18 fat alcohol 7EO. With increasing additive concentration, less protein was adsorbed. Although no statistically significant difference was found between results for different polyvinylpyrrolidone concentrations, protein adsorption tends to diminish with the increasing concentration of the nonionic polymeric dispersant (Figure 5). The addition of both nonionic surfactants similarly reduced protein adsorption of *H. jecorina* cellulase preparation TC (or: Total Crude) from ca. 40 to 50% (0 g l⁻¹) to below 10% (2 g l⁻¹), whereas with polyvinylpyrrolidone the observed effect was only ca. 10%.

Protein adsorption onto a cotton fabric was also investigated for different preparations of *H. jecorina* cellulase. Protein adsorption of the exoglucanase-free EG-rich preparation was lower without additives than that of the whole cellulase preparation TC (or: Total Crude) and, with the addition of 2 g l⁻¹ C10 oxoalcohol 7EO, a significant reduction in protein adsorption was achieved (Figure 6). Even for the CBH-rich preparation (without EGI and EGII) a reduction in protein adsorption was observed (results not shown), which is in agreement with earlier findings [34]. Results indicate that, up to certain limit of additive concentration, protein adsorption decreases with increasing surfactant concentration; however, this concentration limit will depend on enzyme (protein) concentration and on the additive.

Conclusions

All of the tested additives reduced indigo adsorption, but only the use of nonionic surfactants, especially ethoxylated alkylphenols (with 10 and 20EO) and C16-C18 fat alcohols (7EO) were efficient at concentrations as low as 0.2 g l⁻¹. The influence of three additives, two ethoxylated surfactants, a C10 oxoalcohol 7EO and a C16-C18 fat alcohol 7EO, and the nonionic polymer polyvinylpyrrolidone, on the treatment of a plain woven cotton fabric with *H. jecorina* cellulases was investigated and, for all of them, an increase in weight loss after 120 min was observed. The increase in weight loss was more pronounced for treatments with cellulase preparations enriched in EGI and EGII of *H. jecorina*, which suggests that the additives have more influence on endocellulase action than on exocellulase action. A slight increase in the formation of reducing sugars was verified for the nonionic surfactant C10 oxoalcohol 7EO and the dispersant polyvinylpyrrolidone, but possibly longer treatment times would result in more significant differences between treatments without and with additives, as already described for other surfactants with cellulosic and ligno-cellulosic substrates [17,19,24].

All studied additives were found to reduce protein adsorption onto the cotton fabric after 120 min. At a concentration of 2 g l⁻¹ for the nonionic surfactants, a reduction from ca. 40 to 50% to less than 10% of protein was observed, whereas protein adsorption was only slightly reduced by the nonionic polymer polyvinylpyrrolidone. The addition of the surfactant C10 oxoalcohol also showed different effects on different cellulase preparations. For an endoglucanase enriched cellulase the highest surfactant

concentration (2 g L^{-1}) had a more pronounced effect on the reduction of protein adsorption than for a cellobiohydrolase enriched cellulase or a total cellulase mixture. These results support former findings that nonionic surfactants mainly affect endoglucanase–substrate adsorption [17,18] and increased cellulase activity is probably attributable to facilitated protein desorption and less unproductive protein adsorption. Results suggest that the addition of selected additives, such as surfactants and dispersing polymers, during cellulase treatments on indigo and pigment dyed cotton fabrics can have double benefit: first, efficiently reduced back-staining of the dyes and, second, increased cellulase activity on the cellulosic substrate. Reduced protein adsorption to cotton would also diminish the chances of indigo back-staining attributable to the indigo carrier properties of cellulase proteins [9,10], as previously described [6,7].

Acknowledgements

The authors would like to thank Mee-Young Yoon from Genencor for delivering the EGI- and EGII-enriched enzymes, Rhoem Enzymes for all other enzymes, BASF for the surfactant and dispersant samples, and the Brazilian National Council of Scientific and Technological Development (CNPq), Capes (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and the Regional University of Blumenau (FURB) for financial support and providing the grants of Luana Zilz and Martinho Rau.

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