4.3 Degradation and Modification of Cellulose Acetates by Biological Systems

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Summary: A survey is given on recent findings in the enzymology of cellulose acetate degradation. Acetyl esterases have been identified as the principal enzymes, initiating cellulose acetate degradation as a prerequisite for endoglucanase-catalyzed cellulose acetate depolymerisation. Acetyl esterases are provided by nature to deacetylate naturally occurring partly acetylated polysaccharides, i.e. xylan and chitin. Accordingly they are not designed to attack high DS cellulose acetate. Under these circumstances acetyl esterases require a pretreatment of cellulose acetate, leading to some reduction in DS, in case highly substituted material should be degraded. One of these treatments is composting under the conditions of which a partial deacetylation may occur under the action of heat and high pH, facilitating the accessibility for acetyl esterases. However from the present knowledge it cannot be excluded that certain microbial specialists exist, being capable to degrade high DS cellulose acetate.

Keywords: acetyl esterase, endoglucanase, carbohydrate esterase family, NMR-spectroscopy, deacetylation, composting, degree of substitution

4.3.1 Introduction

A variety of test procedures have been developed for the assessment of the biodegradadability of materials and products. Some of these methods are ensuring that material properties are not negatively affected by this feature. Accordingly the durability of plastics against the attack by bacteria and fungi are specified in DIN 53 739[1] and ASTM G 21.[2]

From the standpoint of sustainable raw material cycles and environmental friendly waste management systems biodegradability is considered as a favourable material property. In this

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context biodegradation can principally be tested by measuring the oxygen demand or the carbon dioxide formation. A variety of ISO norms are focussing on these procedures. ISO 14851[3] describes the oxygen consumption in respirator measurements, while ISO 14852[4] and ISO 14855[5] are focused on the determination of evolved carbon dioxide. ISO/DIS 16929[6] specifies the conditions for composting trials under pilot plant conditions, and anaerobic degradation (tested by the release of biogas) is specified in ISO/DIS 14853.[7]

The American standard test methods define the basic specifications of biodegradable materials in ASTM D 6400[8], while the procedures of composting experiments are specified in ASTM D 5338[9] and ASTM D 6002.[10] In Germany the corresponding standard is DIN 54900.[11] Generally the basic principles of test methods are quite similar between different organizations. However the definition of the expected extent in degradation is variing. Recently a continuous harmonisation of specifications and test procedures has been started and will continue in the future. A society for the certification of biodegradable materials has been founded in Germany (DIN CERTCO), which offers a protected label with a logo for compostable materials. The certification scheme is currently accepting the DIN V 54900[11], DIN EN 13432[12] and ASTM D 6400[8] standard procedures, which underlines that the various systems are somehow converging.

4.3.2 Cellulose Acetate Degradation by Microbes and Cell-Free Enzyme Systems

There exists a long going debate on the question, whether cellulose acetates (CAs) are susceptible to biological systems or not. Within their excellent review on cellulose ester performance and application Edgar and associates[13] have also discussed the question on biodegradability of cellulose esters, coming to the conclusion that industrially used cellulose acetates are more or less biodegradable, specially under composting conditions. In earlier publications many authors have clearly stated that CA is not degradable and extremely recalcitrant against microbial attack, e.g. by *Actinomyces*[14], although it has been known for a long time that CA of rather low DS (0.8 ± 0.2) can easily be degraded.[15-16] In another report reverse osmosis membranes made of CA DS 2.5 were incubated with a variety of microorganisms. Several sources of microorganisms were more potential in attacking CA than others. After 2 months incubation some CA samples lost

their semipermeability in salt rejection. Up to 50% loss of the acetyl content had occurred. Cellulose triacetate membranes, however, were resistant to degradation.[17] This experimental finding was supported by Buchanan *et al.*[18] The authors found cellulose acetates with DS values between 1.7 and 2.5 to biodegrade by mixed culture systems under aerobic conditions, at least partly, following deacetylation and depolymerisation. The rate of degradation was highly dependent on the DS. Incubating CA with enriched cultures speeded up the degradation process. A potent CA degrading microorganism seems to be *Rhizobium meliloti*, initiating a 34% weight loss of a CA membrane (DS 2.81) after 150 days. Similarly a CA membrane (DS 2.9) lost 23% of its weight.[19] *Alcaligenes xylosoxidans*, isolated from river water, was identified as another CA degrading microorganism.[20]

The biological degradation of cellulose acetates and cellulose propionates (CP) in an *in vitro* enrichment assay was compared using a series of corresponding cellulose esters between DS 1.8 and 2.6. Within the higher DS range CPs were more resistant to biological deesterification compared to CAs. However rapid degradation was observed, when CP of lower DS (1.77 and 1.84) were incubated (70-80% loss of radiochemical carbonyl labelling after 29 days). Accordingly the accessibility of cellulose esters towards microorganisms is not only dependent on the DS, but also on the acyl chain length.[21] This finding was supported by the research of Glasser and coworkers.[22] The authors synthesized series of other esters than acetates with acyl substituents ranging in size between propionyl and myristyl and DS values between 0.1 and nearly 3.0. They tested their biodegradability not with whole microorganisms, but with cell-free cellulolytic enzymes and also found a dependency on the degree of substitution and substituent size. The maximum degree of acylation, which the enzyme could tolerate before the polymer became undegradable and which resulted in more than 10% degradation by weight, ranged from DS 0.5 to at least 1.0, depending on the ester type. It became evident that the larger the substituent of the cellulose ester, the harder it became for the enzyme to recognize the macromolecule and degrade it.

CA biodegradation was also tested under anaerobic conditions, incubating CA of increasing DS (0.82 to 2.4) with a special culture for an incubation period of 98 days.[23] Only CAs of DS<1.25 were significantly degraded. Samples of DS>1.7 have been degraded to a minor content (16.3 % anaerobic conversion after 90 days.[23] In another study a CA DS 1.7 film was incubated in a

bioreactor under anaerobic conditions. After 30 days the CA film had disappeared completely due to its biological degradation.[24] Unfortunately the outcome of the experiment with a corresponding DS 2.5 CA film was not reported. The authors stressed the point that not only aerobic but also anaerobic microorganisms are known to produce the complete set of hydrolases including esterases, necessary to degrade naturally occurring acetylated plant polysaccharides, e.g. acetylxylan. The anaerobic degradation of cigarette filter tips was also verified in the work of Zenjian *et al.*[25]

CA films of DS 1.7 and 2.5 were also exposed under composting conditions, which can be defined by an aerobic biological process involving a succession of rapidly changing microorganisms.[26] In the initial stages of composting usually the pH starts in the neutral range, drops slightly into the acid pH-range followed by a rise up to pH 8-9. The temperature may rise up to 70 °C and higher.[27] In the composting industry these conditions are highly welcome as a prerequisite for soil sterilisation. These are also conditions, under which, at least partly, acetyl substituents start to be cleaved off. A DS 1.7 CA film was completely disintegrated after 7 days composting, whereas 18 days composting were required for the DS 2.5 CA film to disappear.[26] From these results it becomes apparent that the succession of different microorganisms with an extremely large variety of different individual enzyme systems, paired with conditions under which part of the acetyl substituents start to be cleaved off might be the reason, why composting seems to be rather efficient in the biological breakdown of cellulose acetate. Specially this is the case, when composting is compared to incubation with single microorganisms or mixes thereof. However it cannot totally be excluded that certain microbial systems besides hydrolases exist, which are capable to degrade CA of high DS.

4.3.2.1 The Cellulolytic Enzyme System

Complete cellulose hydrolysis requires the action of exoglucanases (also called cellobiohydrolases, CBH), endoglucanases (EG) and ß-glucosidases. Not all microorganisms produce the complete set of cellulases for efficient degradation of insoluble cellulose. Especially CBHs are lacking some of them. The general term "cellulase" usually refers to the complete set of cellulolytic enzymes. According to the current hypothesis EGs initialise the attack on cellulose by randomly hydrolyzing internal bonds in amorphous cellulose regions. EGs thus produce new

chain ends, which then become available for CBHs, attacking the chains from the reducing (CBH I) and non-reducing end (CBH II), whereby cellobiose is liberated.[28-29] Finally ß-glucosidases hydrolyze cellobiose into glucose. The different cellulases have been shown to hydrolyze cellulose synergistically.

The proposed degradation pathway as well as the synergy has been subject of extensive discussion during the recent years. Thus the present classification of specific cellulases being either CBH or EG seems dubious. Today cellulases are classified for their amino acid sequence, followed by a classification by means of hydrophobic cluster analysis, HCA, in addition to their action against specific substrates.[30] The HCA considers the different charges of amino acids and identifies hydrophobic clusters along the two dimensional sequences. Based on this a three dimensional structure can be predicted. The method is especially useful for the detection of similar folds in different enzymes with low sequence identity.

Within the currently 91 classified glycoside hydrolase (GH) families 13 "clans" of related families are identified.[31] EGs (EC 3.2.1.4) are found in 14 GH-families, CBHs (EC 3.2.1.91) in 5. In all of these CBHs containing GH-families endoglucanases are found as well.

The first true exoglucanase structures solved are the two CBHs of the fungus *Trichoderma reesei*. The three-dimensional structure of the catalytic domain of *T. reesei* CBH II (family 6) and CBH I (family 7) revealed that the active site of both enzymes was identified to be situated in a tunnel ranging through the whole domain and formed by stable surface loops.[32-33] It therefore seems that the active site tunnel is a general feature of exoglucanases. Indeed exoglucanases are believed to be unable to attack cellulose derivatives and the existence of the active site inside a tunnel could explain that especially bulky cellulose derivatives would get stuck inside the tunnel. This implies that EGs should have similar overall folds, but the active site should be more open allowing a random hydrolysis of the cellulose.[32] The three-dimensional structures of several enzymes have confirmed this hypothesis.[34-35]

The reaction mechanisms vary between the GH-families. Both, inverting as well as retaining of the configuration is common between the GH-families.[31]

Davies and Henrissat[36] compared all the solved structures and came to the conclusion that the active site topologies can be divided into three classes (Fig. 1). Pocket-like active sites are found in enzymes, cleaving monosaccharides from chain ends, such as ß-glucosidases (Fig. 1A).

The second class of topologies includes active sites, situated in a tunnel, which allows the enzyme to remain bound to the cellulose chain, while releasing products from the chain ends. Examples of this class are CBH I and CBH II (Fig. 1B), which are responsible for the degradation of crystalline cellulose. It is near at hand that both cellobiohydrolases are somehow restricted in their action when the substrate, passing the tunnel, is made of cellulose derivatives instead of native cellulose. The third class of topologies includes active sites situated in a groove or a cleft, open from both ends (Fig. 1C). This open arrangement allows the enzyme to bind to its substrate in a random manner and is found in endo-acting enzymes like endoglucanases. The open active site structure is rather helpful, in case cellulose derivatives should be cleaved into pieces.

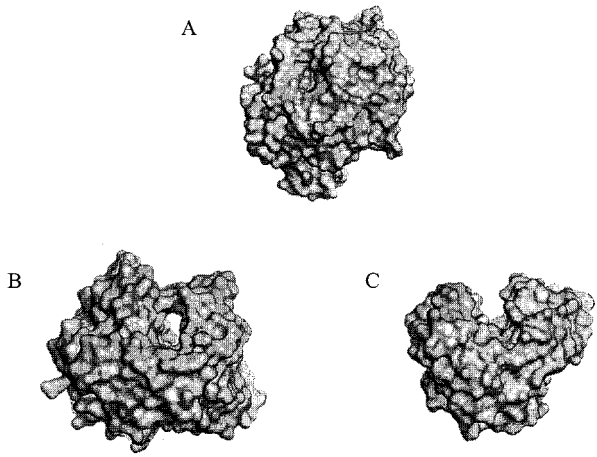

Figure 1. Active site topologies of glycosyl hydrolases. **A**: The pocket of a glucoamylase. **B**: The tunnel of CBH II of *T. reesei*. **C**: The cleft of Endoglucanase 2 of *T. fusca*. From Davies and Henrissat.[36]

Cellobiose-Quinonoxidoreductase (CBQ) and Cellobioseoxidase (CBO) are additional cellulolytic enzymes, being produced by white-rot fungi. The role of both enzymes in cellulose as well as lignin degradation is not so well recognized.[37] These enzymes oxidize cellobiose to cellobiono-1,5-lactone and reduce phenoxy-radicals, which have been produced by laccase and lignin peroxidases during lignin degradation. CBO also reduces Fe(III). In the presence of

hydrogen peroxide, which is also produced by CBO, Fenton's reagent may be formed, initiating the production of hydroxy radicals. The latter substances attack both, cellulose and lignin.

4.3.2.2 Cellulose and Cellulose Acetate Degradation by Cellulolytic and Non-Cellulolytic Enzymes

Cellobiohydrolases I and II are the principal enzymes responsible for cellulose hydrolysis. However due to their active site topologies, situated in a tunnel these important enzymes are rather restricted in the hydrolysis of CA, even in case the DS is rather low. This is demonstrated by the incubation of both enzymes, single and in synergy, with DS 0.7 CA fragments of DP between 9 and 17 (Fig. 2 bottom right). After deacetylation the reference material is degraded into cellobiose rather rapidly (not shown here). However the DS 0.7 acetylated cellulose fragments turned out to be rather resistant against the enzymatic attack from the reducing end by CBH I (Fig. 2 top left) and from the non-reducing end by CBH II (Fig. 2 bottom left). Although

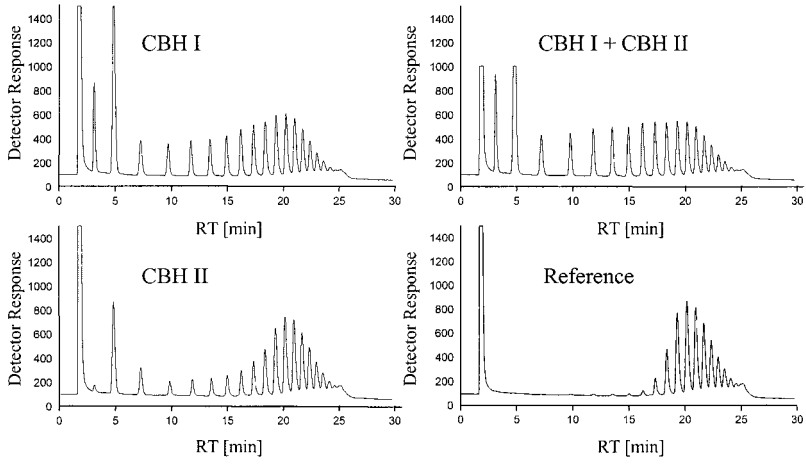

Figure 2. HPAEC chromatography of CA DS 0.7 fragments after incubation with CBH I and II, single and in synergy. Retention time (RT) of degradation products: cellobiose 5 min.; cellotetraose 10 min.; celloheptaose = 15 min.; celloduodecaose 20 min. Separation column: CarboPac PA1 (4x250 mm, Dionex Corp.).

both enzymes acted in synergy from both ends a substantial amount of the initial reference material (DP 9 –17) survived the enzymatic attack (Fig. 2 top right). It is demonstrated in Fig. 2 that the principal degradation product of both enzymes is cellobiose, being eluted from the HPLC-column after 5 min.

Certain similarities in the enzymology of cellulose and hemicellulose degradation are already known for a longer time, since endoglucanase I is not only degrading amorphous cellulose, but also able to depolymerise xylan.[38] Furthermore there exist acetyl esterases, which not only deacetylate the naturally occurring acetylxylan, but also cellulose acetate in the range between DS 0.7 to 1.6.[39] In an earlier publication the same authors have reported on a wide distribution of acetyl esterases among industrial enzyme preparations, being capable to deacetylate low DS CA. Cellulosetriacetate seems to be inert against common enzymes due to the lack of hydrophilicity and obstruction by a larger number of bulky substituents.[40-41] However in case part of the acetyl groups have been cleaved off, e.g. under the influence of an appropriate pH and temperature during composting, acetyl esterases may efficiently start to deacetylate more substituents in order to smoothen the accessibility for endoglucanases.

The synergistic action of acetyl esterase and endoglucanase has been demonstrated by incubation of a mono-component *Aspergillus* endoglucanase and an enzyme mix including endoglucanase and acetyl esterase activity. The incubated samples were directly analysed by aqueous size exclusion chromatography (SEC). The time course of the degradation is illustrated by the elution

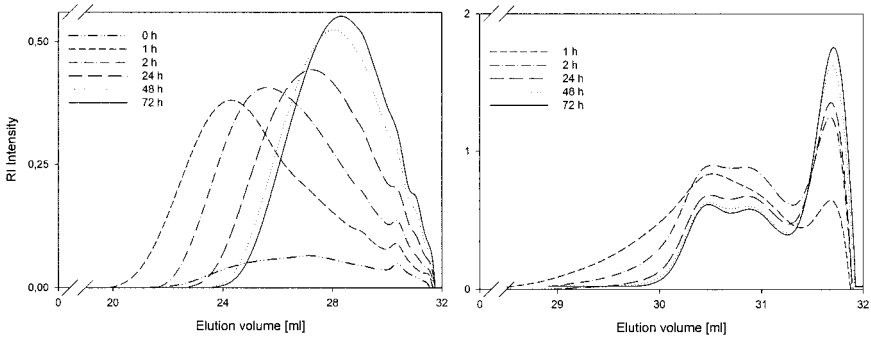

Figure 3. Time course of elution profiles from size exclusion chromatography of cellulose acetate (DS 0.7) after degradation by mono- component endoglucanase (left) and endoglucanase / acetyl esterase (right). From [42].

curves from 0 to 72 hours incubation (Figure 3). The improving RI intensity parallel with the increasing degradation rate, special in the comparison of the substrate blank (0 h) with the sample after 1 hour incubation, can be deducted to the improved water-solubility of the material. The shift to smaller fragments equivalent with longer elution times is particularly obvious within the first 24 hours of incubation. There was nearly no change between 48 hours and 72 hours of incubation. The substrate blank was not included in Figure 3 (right) in order to allow an improved resolution of the low molecular weight fragment (note the different ml-scale in both profiles of Figure 3). Indeed the polymeric material was already drastically reduced in chain length after one hour of incubation. After 24 hours only minor additional changes occurred. This difference in speed and extent of degradation has been made possible by the presence of an acetyl esterase in addition to the endoglucanase activity. Unambiguously the presence of this enzyme was established by the release of acetic acid into solution.[42]

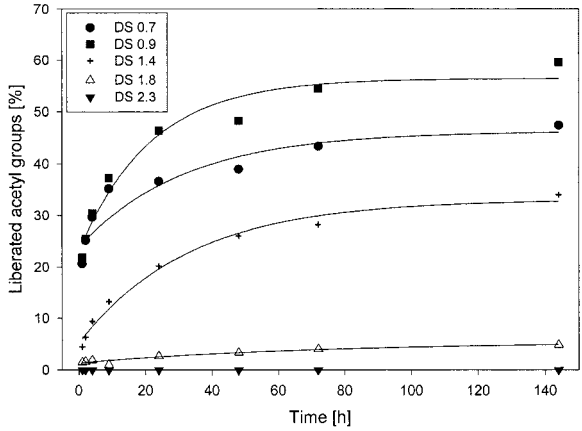

Figure 4. The effect of CA degree of substitution and reaction time on acetic acid release by a CE family 1 acetyl esterase from *Aspergillus niger*. From [39].

The first purification of an esterase from *Neisseria sicca*, involved in cellulose acetate degradation was reported by Moriyoshi *et al.*[43] The enzyme catalysed the hydrolysis of acetyl saccharides and p-nitrophenyl esters of short-chain fatty acids. Altaner *et al.*[39] isolated an acetyl esterase from *Aspergillus niger*, involved in CA break-down. The enzyme could deacetylate CA

up to DS 1.8 to a certain extent, showing a decrease in activity with increasing sample DS (see Fig. 4). The DS 2.3 CA was not a substrate of this *Aspergillus niger* enzyme.

It deacetylated CA independent from other cellulolytic enzyme action, however the acetyl esterase supported endoglucanases in depolymerising CA.[39] The accessibility of the acetyl esterase towards CA of higher DS was not improved in the presence of any cellulolytic enzyme. Accordingly acetyl esterases seem to be the most important class of enzymes in CA biodegradation. The *Aspergillus niger* acetyl esterase was also very active on native acetylxylan (DS 0.4-0.5), whereas the activity against starch acetate (DS 0.6) was very low. Cellulose sulphate was no substrate of this enzyme. Based on its N-terminal amino acid sequence the enzyme could be identified as true acetylxylan esterase, belonging to CE family 1, as described in the CAZY database.[31] Typical acetylxylan esterases, as described in the literature, belong to the same family.[44-45] However acetylxylan esterases occur in CE families 1 to 7 within the currently 13 classified CE families. In a comparative experiment acetylxylan esterases from CE families 1, 4, and 5 as well as two non-classified acetylxylan esterases have been incubated with CA DS 0.7. As the outcome of this experiment CA was a substrate for all of them, although there were differences in the extent of deacetylation between the acetyl esterases from different families.[46] All these enzymes exhibited a three layer (α/β/α)-sandwich fold with a classical Ser-Asp-His catalytic triad as active site. In a similar comparative experiment two CE family 4 enzymes, namely a chitin deacetylase from *Mucor rouxii* and an acetylxylan esterase from *Streptomyces lividans* have been examined.[47] Both enzymes were active against acetylxylan and several soluble chitinous substrates, whereas an acetylxylan esterase from *Bacillus pumilus*, classified under CE family 7, was found to be inactive towards all chitinous substrates tested. Since the acetylxylan esterases from CE families 1,4, and 5 were active against CA, it is near at hand that the CE family 4 chitin deacetylase from *Mucor rouxii* would also deacetylate cellulose acetate. A closer look to the conformation of the artificially and naturally occurring acetylatated polysaccharides chitin, acetylxylan and cellulose acetate reveals that the acetyl substituents of these substrates are identically arranged in the aequatorial plane (Fig. 5). Accordingly it would be conceivable that the active site of the same enzyme, specialized for deacetylation of the acetylxylan and chitin would also be able to accommodate CA, in case the CA DS is more or less similar to the DS of chitin (DS 1) and/or acetylxylan (DS 0.4-0.7).

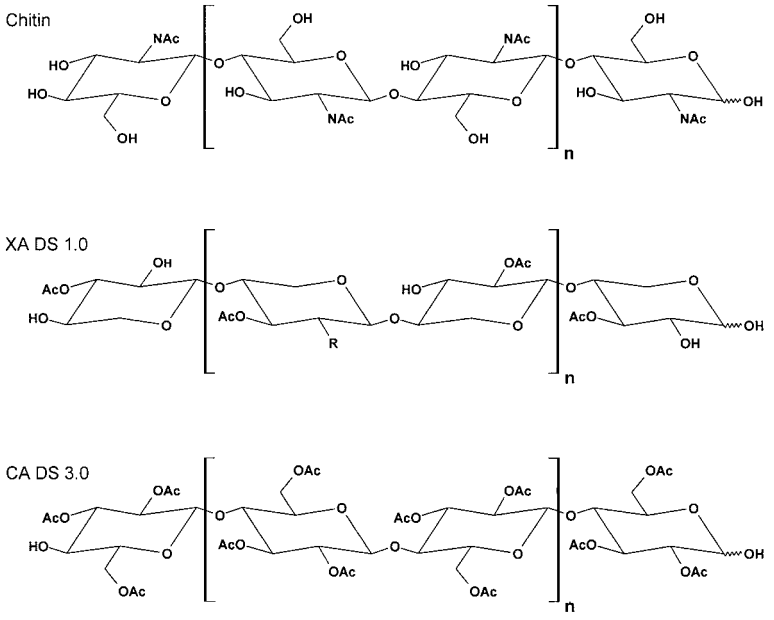

Figure 5. Arrangement of substituents in naturally and artificially acetylated polysaccharides: Chitin, acetylxylan and cellulose acetate. Xylan substituents R vary dependent on the xylan source (arabinofuranosyl or 4-0-methylglucuronosyl).

4.3.3 Cellulose Acetate Degradation by UV Irradiation

The difficulties in promoting CA degradation by microbial systems alone have been stated quite often. It has also been suggested that UV irradiation would play an important role in further degradation of CA in the natural environment. Indeed it could be demonstrated that UV irradiation resulted in an increase of the surface area of CA films.[48] From NMR and SEC results the authors could clearly show that UV irradiation only slightly lowered the DS, but lowered the molecular weight (Mw) of CA to less than one-tenth of the initial Mw. It can be concluded that the slight decrease in DS would not improve the accessibility of CA to cellulases. However UV irradiation would initiate a partial solubilization of the surface area of CA film, resulting into an

increased surface area and an increase in contactabiliy with microbes and their enzymes.[48] In order to accelerate the photodegradation of CA it has been proposed to add pigments like titanium dioxide in quantities between 0.05 and 5% by weight, which act as photooxidation catalysts.[49-50] The photoactivity of the titanium dioxide is dependent upon crystal type, with anatase being more photoactive than rutile. In addition particle size and any dispersion-enhancing coatings will influence the photodegradation rate.

4.3.4 Targeted modification of cellulose acetates

In a continued study possible differences between the acetyl esterases, grouped into the particular CE families were investigated by ^{13}C-NMR spectroscopy after incubation of cellulose acetate DS 0.7 (Fig. 6). The spectrum of the reference sample CA DS 0.7 (Fig. 6 top) demonstrates that the signals of the substituents in C3-position were the best resolved, and the 3-mono, 2,3-di, 3,6-di and 2,3,6-tri-*O*-acetyl anhydroglucose units (AGUs) could be distinguished according to the assignment of Buchanan *et al.*[51]

The CE 1 family esterases, e.g. the esterase from *Aspergillus oryzae* exclusively deacetylated CA in the C2- and C3-positions, resulting in a regioselectively C6-substituted CA. The NMR-measurement of the sample, incubated with the CE 4 family acetylxylan esterase from *Streptomyces lividans* revealed that this enzyme exclusively reduced the number of acetyl substituents in C3-position (Fig. 6). In this deacetylation stage exclusively the signal of the 3-mono substituted AGUs were reduced, while the C3-signals of the 2,3-di-, 3,6-di and 2,3,6-trisubstituted AGUs persisted. The CE 5 family enzyme acetylxylan esterase II from *Penicillium purpurogenum* deacetylated CA only in the C2-position, leaving the C3- and C6-acetyl groups attached (Fig. 6 bottom). The enzyme attacked substituents in C2-position regardless of the fact, whether the AGU was tri-, di- or mono-substituted. This can also be assumed, because it seemed that the 2,3,6-tri signal was reduced while the 3,6-di signal was increased. Due to the different regioselective modes of action found for esterases of different CE families, they can be used for the production of regioselectively substituted CA. With the help of CE 1 and CE 5 family esterases, regioselectively 6-O-acetylated and 3,6-O-acetylated CA, respectively, could be prepared, while CA can be regioselectively modified in C3-position with the CE 4 family

enzyme.[46] Regioselectively substituted cellulose esters have also been obtained by enzyme-catalyzed transesterification. Subtilisin Carlsberg was found to be catalytically active in the transesterification of cellulose with vinyl propionate and vinyl acrylate in anhydrous pyridine.[52]

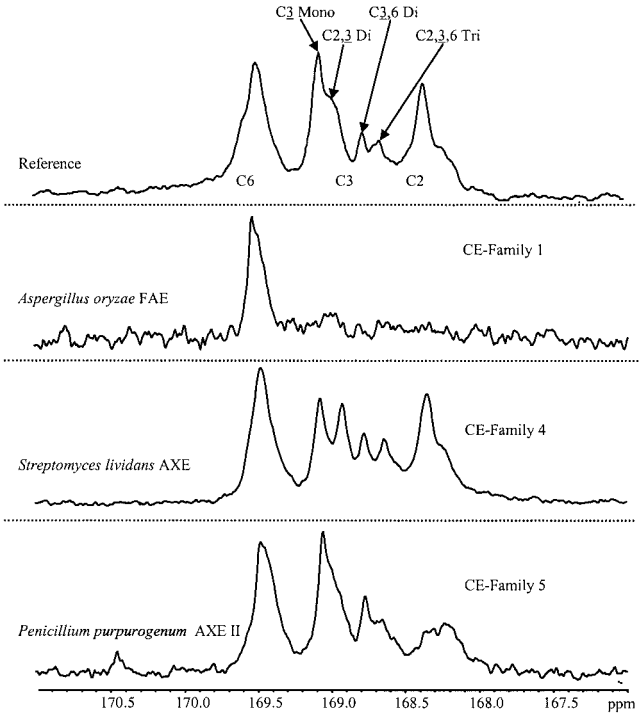

Figure 6. Carbonyl range of the ^{13}C-NMR spectra of cellulose acetate DS 0.7 incubated with CE-family 1, family 4 and family 5 acetylxylan esterases. From [46].

4.3.5 Conclusion

Cellulose acetate of DS<2.0 can be degraded by cell-free enzymes. In this process acetyl esterases play the predominant role, acting independently from endoglucanases. Acetyl esterases are not foreseen by nature to degrade CA, but to deacetylate naturally occurring and partly acetylated polysaccharides, e.g. chitin, mannan and xylan. Due to the same arrangement of acetyl

substituents in xylan, chitin and CA acetyl esterases recognize partly acetylated CA as a substrate, which they can accommodate within their active site. Since xylan is missing a C6-position it is clear that acetyl substituents at this position cannot be removed. Recent work has demonstrated that there exist a further specialisation among acetyl esterases. This may be explained by differences in the position of acetyl substituents in their naturally occurring substrates. Certain acetyl esterases cleave off the substituents from the C2- and C3-position (CE family 1 enzymes), whereas others only deacetylate substituents from the C2-position (CE family 5 enzymes) or from the C3-position (family 4 enzymes). This phenomenon can be used to manufacture regioselectively substituted CA of low DS. As a prerequisite for acetyl esterase action, CA must be deacetylated to a certain extent, which may be achieved by the action of heat and alkalinity under effective composting conditions. At present there exist no data on the involvement of non-hydrolytic enzyme systems for cellulose acetate biodegradation.

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