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Enzymatic Degradation of Cellulose-Based Materials

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The biodegradability of cellulose-based materials was compared in the standard Sturm test and by enzymatic hydrolysis. *Trichoderma reesei* culture filtrate, the purified enzymes endoglucanase I and II from *T. reesei*, and β-glucosidase from *Aspergillus niger* were used in the experiments. The unpurified *Trichoderma reesei* culture filtrate was found to contain a mixture of enzymes suitable for cellulose degradation. However, when purified enzymes were used the right balance of the individual enzymes was necessary. The addition of β-glucosidase enhanced the enzymatic hydrolysis of cellulose materials when both culture filtrate and purified enzymes were used. In the Sturm test the biodegradability of most of the cellulose materials exceeded 70% carbon dioxide generation, but, in contrast, the biodegradability of the highly substituted aminated cellulose and cellulose acetate was below 10%. The results concerning enzymatic hydrolysis and biodegradability were in good agreement for kraft paper, sausage casing, aminated cellulose, and cellulose acetate. However, diverging results were obtained with cotton fabric, probably as a result of its high crystallinity.

KEY WORDS: Cellulases; biodegradability test; Sturm test; packaging materials.

INTRODUCTION

Cellulose-based packaging materials account for a high proportion of all packaging materials. During processing the cellulose is modified, e.g., in pulping, bleaching, and cardboard manufacturing. Various additives are also frequently added in paper making and during the finishing processes. Owing to the hydrophilic nature of the paper fibers, coating the material with waxes, plastic, etc., is common.

The crystallinity and structural organization of cellulose vary according to its origin and processing [1]. The morphology of the crystalline structure of the polymer is known to affect biodegradation. Cellulose can be chemically modified, e.g., as in the case of cellulose acetate, in order to improve and modify the technical properties of the material. Chemical modification of cellulose affects its biodegradation. Of the cellulose derivatives, cellulose acetates have been studied the most, and the high degree of substitution of cellulose esters has been shown to decrease their biodegradability [2–4].

Cellulose is a renewable carbon source consisting solely of glucose units, and it is degraded by extracellular enzymes excreted by various microbes. Fungi, including *Trichoderma*, *Penicillium*, and *Fusarium* spp., are efficient producers of cellulolytic enzymes [5]. Cellulose, although chemically simple, is structurally a complex polymer and several enzymes are needed for complete degradation of this material [6].

Trichoderma reesei has an efficient and well-characterized cellulase system (see, e.g., Ref. 7). In addition, it has an exceptionally high excretion capacity and it is therefore used as an industrial source of cellulases. The major cellulases of *T. reesei* are cellobiohydrolases I and II and endoglucanases I and II, the genes for which have been characterized. The genes coding for at least two minor endoglucanases have also been identified [8, 9]. The cellulase system consists predominantly of products from the four major genes. Due to posttranslational modification and proteolysis, however, the culture filtrates

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typically contain more than 10 forms of the major cellulases with respect to their isoelectric point and molecular weight. The culture filtrates of *Trichoderma* sp. generally contain relatively small amounts of β-glucosidase [10]. This enzyme is, however, essential for the complete hydrolysis of cellulose. Some of the soluble hydrolysis products are known to inhibit action of the cellulases, and hydrolysis can usually be accelerated by decreasing the amount of cellobiose in the hydrolysis mixtures by the addition of β-glucosidase.

Biodegradation is defined according to CEN (European committee for standardization) as the degradation of packaging material mediated by a biological system, such as enzymes. Standard biodegradability tests are laborious and time-consuming, because they are based on the microbial degradation of polymers either under aquatic conditions with sewage sludge microorganisms or under thermophilic composting conditions. Most of the tests are based on the measurement of CO_2 evolution resulting from polymer biodegradation [11].

Hydrolysis is the principal mechanism by which enzymes degrade cellulose polymers. The first steps in depolymerization take place outside the microbial cells through the action of extracellular enzymes. After cleavage, the smaller oligomers can be transported into the cells for final mineralization [12]. An enzymatic degradation test has been successfully used in our earlier studies on starch-based materials to evaluate biodegradability [14]. In this study the biodegradability of cellulose-based products was studied using a fungal culture medium containing a complete set of enzymes, by a mixture of purified cellulases, as well as by the standard Sturm test.

MATERIALS AND METHODS

Cellulose Samples

The cellulosic materials studied were filter paper (Whatman, No. 1), cellulose film (Cellophane 325 P prepared from viscose cellulose by Walki, Yhtyneet Paperitehtaat, Valkeakoski, Finland), sausage casing (PRR130; Oy Visko Ab; 111 g m^{-2}; a composite of abaca fiber and viscose cellulose), cellulose acetate (Fluka 22194; mean molecular mass, ca. 61.000; DS 2.5), aminated cellulose (prepared from bleached kraft pulp by VTT Chemical Technology; nitrogen content, 4.0%), and unbleached kraft paper manufactured for packaging material (Wisaforest 6388/56678; 70 g m^{-2}). Cotton fabric (140 g m^{-2}) which had been desized by α-amylase treatment was obtained from Finlayson Oy (Tampere, Finland).

The samples were ground cryogenically in an ultracentrifuge mill (Retch ZM1; Germany) with a 0.5-mm sieve. The ground material was used in the hydrolysis studies and biodegradation experiments, unless otherwise described.

Assays

β-Glucosidase was assayed with 4-nitrophenyl β-D-glucopyranoside as substrate [15]. Protein was assayed by the method of Lowry *et al.* (1951) [16] using bovine serum albumin (Sigma) as the standard.

Enzymes

A culture filtrate of *Trichoderma reesei* Rut C-30 (VTT-D-86271) was obtained from a pilot-scale fermentation, treated with bentonite, and concentrated as described earlier [17]. The concentrated culture filtrate containing all the major cellulases of *T. reesei* and a wide range of other hydrolytic enzymes in minor concentrations is hereafter referred to as the culture filtrate. Endoglucanases (EG) I and II and cellobiohydrolases (CBH) I and II from *Trichoderma reesei* were purified as described earlier [17]. β-Glucosidase (from *Aspergillus niger*) was obtained from Megazyme (Australia) or from Novo Nordisk (Novozym 188; Denmark).

Hydrolysis Experiments

In order to compare the purified cellulases with the culture filtrate, three unsubstituted cellulosic materials (filter paper, unbleached kraft paper, and cotton fabric) were incubated with purified EG I, CBH I, and the culture filtrate. The enzymes were used alone or in combinations, either with or without additional β-glucosidase. The materials were cut into pieces of ca. 1 cm^2 and incubated with shaking for 24 h at 50°C in 20 m*M* sodium acetate buffer, pH 5.0. The dry weight of cellulose in the incubation mixtures was 5 g L^{-1}. The enzyme dosages based on protein concentration were 4 mg g^{-1} cellulose for EGI and CBH I alone, 2 mg g^{-1} cellulose for EG I and CBH I each when used together or with the culture filtrate, and 5 mg g^{-1} cellulose for the culture filtrate. When β-glucosidase was added the dosage was 100 nkat g^{-1} cellulose. The reducing sugars formed in the treatment were assayed by the method of Sumner and Somers [18], using glucose as the standard.

The enzymatic degradation of the cellulosic materials and cellulose derivatives was further studied using

three cellulase mixtures: culture filtrate, culture filtrate supplemented with β-glucosidase, and purified cellulases supplemented with β-glucosidase. The purified cellulases were used as a mixture, prepared by mixing purified enzymes at ratios of 6:2:1:1 (based on protein content) for CBH I:CBH II:EG I:EG II, respectively. The proportions of individual cellulases in this mixture were close to the natural ratio in the culture filtrates of *T. reesei* [19]. The finely ground materials (cellulose film, cotton fabric, unbleached kraft paper, sausage casing, cellulose acetate, and aminated cellulose) were suspended in 20 m*M* sodium acetate, pH 5.0. The dry weight of the suspensions was 2.5 g L^{-1}. The enzymes were added and the blank samples were withdrawn immediately. The suspensions were then incubated for 6 h at 50°C. Samples were also taken after 2 h of hydrolysis. The enzyme dosages, based on protein concentration, were 10 mg g^{-1} cellulose for both the cellulase mixture and the culture filtrate. The dosage of the supplemented β-glucosidase was 200 nkat g^{-1} cellulose. The samples were assayed for reducing sugars as described above.

Biodegradation Experiments

The carbon content of the samples was determined by a Carlo Erba carbon analyzer (NA 1500; Carlo Erba Instruments, Milano, Italy), and 200 mg of the sample calculated as carbon was added to 1000 ml of mineral nutrient medium. Sewage sludge was used as inoculum, prepared as described earlier [20].

Biodegradation of the samples was studied by the Sturm test (ASTM D5209, OECD 301B), which is an aquatic biodegradability test. A constant air flow was maintained through the vessels and the CO_2 evolved as a result of microbial degradation of the polymer was trapped in a 0.1 *M* potassium hydroxide solution. Cumulative evolution of CO_2 was quantitated by an automated conductivity-based method [21] and additionally titrated at the end of the study according to the standard. The inlet air was pushed through the CO_2 traps containing soda lime (Durasorb; Engström mie, Devon, UK). Incubation was continued at +25°C in the dark until microbial growth reached the plateau phase. Two replicates of each sample, and the blank without the cellulose sample but with microbial inoculum, were studied. The mean values of these parallels were calculated and the blanks subtracted to give the final results. A blank containing only the mineral medium was also included in order to monitor the carbon dioxide trapping efficiency of soda lime. The degree of biodegradation of the samples was calculated, according to the standard, on the basis of the amount of carbon dioxide evolved as a percentage of the theoretical amount.

RESULTS AND DISCUSSION

Hydrolysis by Purified Enzymes

The degradability of filter paper, kraft paper, and cotton fabric by different enzymes was first compared. The hydrolysis of these materials was incomplete when single purified cellulases were used (Fig. 1). Simultaneous addition of endoglucanase and cellobiohydrolase enzymes significantly enhanced the hydrolysis of filter paper as a result of synergistic action. Cotton, however, being a highly crystalline material, remained almost unhydrolyzed. As expected, the addition of β-glucosidase to endoglucanase and cellobiohydrolase markedly increased the hydrolysis rate of the cellulose materials. The effect of β-glucosidase addition to the culture filtrate was surprisingly large, indicating that in this case the culture filtrate also contained β-glucosidase activity in insufficient amounts. Consequently, when rapid and advanced hydrolysis was desired, the complete enzyme system with the addition of β-glucosidase in adequate dosages seemed superior.

Enzymatic Degradability of Cellulosic Materials

The chemically unmodified cellulosic materials were readily digested by cellulases, either from a mixture of purified enzymes (Table I) or from the culture filtrate (Table II). The degree of hydrolysis was dependent on the enzyme system used; the addition of β-glucosidase to the culture filtrate especially was essential to obtain rapid enzymatic hydrolysis (Table II).

The substrates with a lower crystallinity were rather readily degraded by purified enzymes, whereas the hydrolysis of the more crystalline cotton was slightly enhanced by the culture filtrate. This can be explained by the fact that the cellulase ratio may be more optimal in the culture filtrate than in the artificial mixture. The other minor cellulolytic proteins in the culture filtrate may also have enhanced the hydrolysis of cotton. In addition, the culture filtrate contained other proteins, e.g., hemicellulases. These additional components may have been advantageous when the sample contained carbohydrate polymers other than cellulose. This was emphasized in the hydrolysis of unbleached kraft paper which contained xylan (glucose:xylose, 100:11.3) and glu-

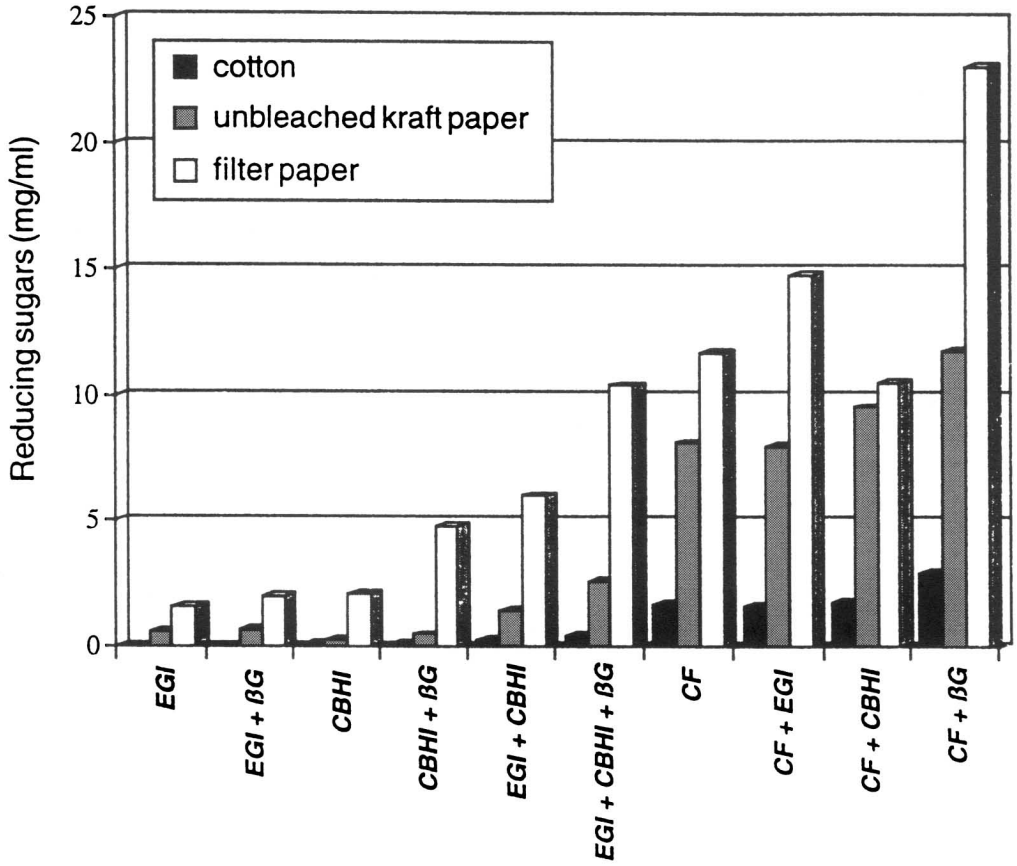

Fig. 1. Release of reducing sugars from cotton, unbleached kraft paper, and filter paper by combinations of purified cellulases of *T. reesei* and β-glucosidase. For dosages and other experimental conditions, see the text. EGI, endoglucanase I; βG, β-glucosidase; CBHI, cellobiohydrolase I; CF, culture filtrate.

comannan (glucose : mannose, 100 : 6.5). The xylanase and mannanase activities present in the culture filtrate enhanced the degradation of these minor constituents, which was seen as a higher final hydrolysis degree (Tables I and II). The cellulase acetate was totally unaffected by the enzymes of *T. reesei*, and the hydrolysis of the other substituted (aminated) cellulose was also negligible.

The materials tested contained some additives other than carbohydrates (e.g., glycerol in the sausage casing). Another hydrolysis-limiting factor for cellulases is the presence of small amounts of lignin. Thus, complete enzymatic hydrolysis was not considered feasible. The hydrolysis degrees obtained within 6 h by this enzyme dosage were high enough to allow comparison of the enzymatic degradability of different samples. The enzymatic degradability decreased in the order sausage casing > cellophane > unbleached kraft paper > cotton fabric > substituted celluloses (Tables I and II).

Table I. Hydrolysis of the Test Materials by a Mixture of Purified Cellulases (CBHI:CBHII:EGI:EGII = 6 : 2 : 1 : 1)[a]

Material	Hydrolysis rate (%)	
	In 2h	In 6h
Cellophane	37	78
Cotton fabric	19	31
Unbleached kraft paper	9	43
Sausage casing	24	82
Aminated bleached kraft pulp	0.07	0.18
Cellulose acetate	0.00	0.00

[a]The hydrolysis rate is given as a percentage of the theoretical maximum value.

Table II. Hydrolysis of the Test Materials by a Culture Filtrate With and Without Additional β-Glucosidase[a]

Material	Hydrolysis rate (%)			
	In 2h		In 6h	
	CF	CF + βG	CF	CF + βG
Cellophane	23	31	41	52
Cotton fabric	10	22	16	34
Unbleached kraft paper	12	26	21	47
Sausage casing	32	46	49	74
Aminated bleached kraft pulp	0.00	0.04	0.07	0.14
Cellulose acetate	0.00	0.00	0.00	0.00

[a]The hydrolysis rate is given as a percentage of the theoretical maximum value. CF, culture filtrate; βG, β-glucosidase.

Biodegradation in the Sturm Test

Cellophane, kraft paper, sausage casing, and cotton fabric were biodegraded rapidly in the Sturm test, evolving more than 60% of the theoretical carbon dioxide amount in the sample within 10 days (Figs. 2b and c and 3b and c). The biodegradability of these materials, calculated as the proportion of carbon dioxide evolved during the test out of the theoretical amount, was 79, 77, 70, and 74%, respectively, when determined by titration. The conductivity-based results are shown in Figs. 2a–c and 3a–c. The final conductivity-based results for cellophane and kraft paper were slightly lower than the results determined by titration. However, the overall results were in good agreement with the two different CO_2 measurements. The conductivity-based measurement was sensitive to small temperature changes, as shown by the fluctuation in the curves (Figs. 2 and 3). The background carbon dioxide, evolving from the inoculum without an additional carbon source, was subtracted from the results shown in Figs. 2a–c and 3a–c. The decreasing biodegradation in the conductivity-based method for some samples was probably due to the higher background CO_2 level compared to that in the actual samples.

The cotton fabric, sausage casing, and kraft paper biodegraded within 10 days in the Sturm test. Cellophane had a longer adaptation phase and also a slower degradation rate. This result was contradictory to those obtained by the enzymatic hydrolysis test, where cellophane was readily and rapidly hydrolyzed by purified enzymes and the culture filtrate. The cellulose derivatives, cellulose acetate and aminated cellulose, were not biodegradable, both evolving less than 10% of the theoretical amount

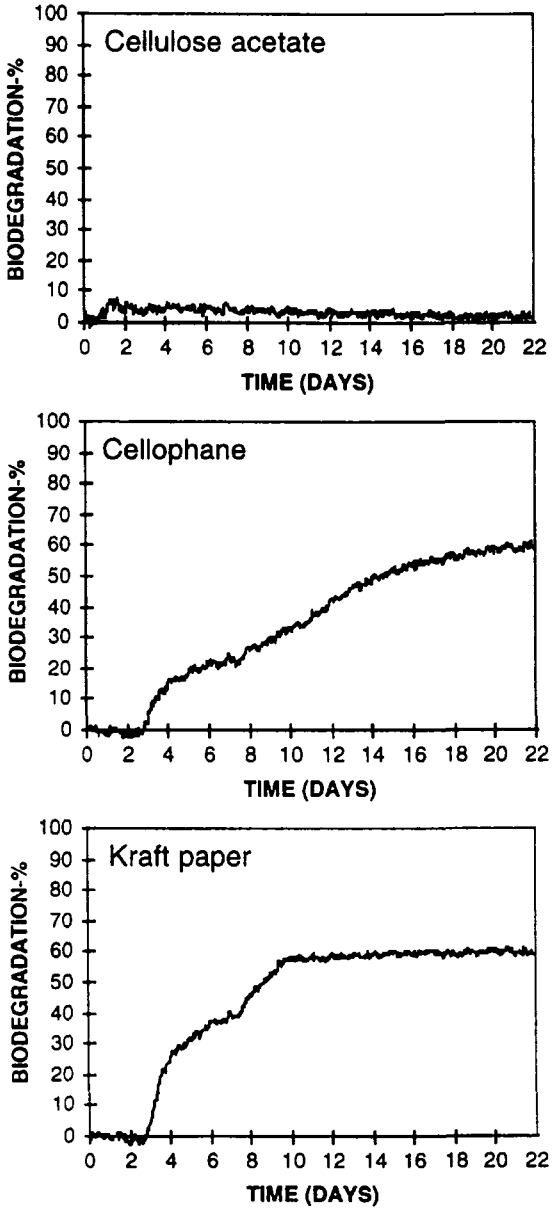

Fig. 2. Biodegradation of (a) cellulose acetate, (b) cellophane, and (c) kraft paper in the Sturm test. Carbon dioxide evolved as a percentage of the theoretical amount in the sample is presented as biodegradation-%.

of CO_2 (Figs. 2a and 3a). The two chemically modified cellulose materials were highly substituted, the degree of substitution being above 2.5, which, according to earlier studies, is known to limit the biodegradation of cellulose acetates [3, 4]. The pass level for the potential biodegradation of chemicals according to OECD Guidelines (1993) is 60% of the theoretical for tests which

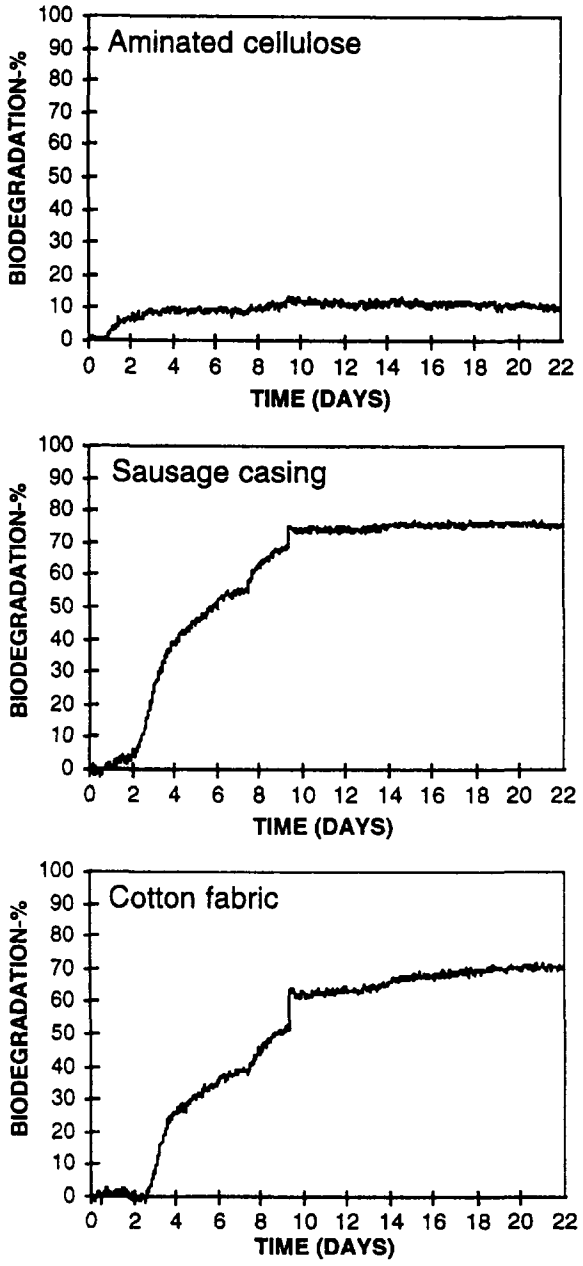

Fig. 3. Biodegradation of (a) aminated cellulose, (b) sausage casing, and (c) cotton fabric in the Sturm test. Carbon dioxide evolved as a percentage of the theoretical amount in the sample is presented as biodegradation-%.

are based on carbon dioxide evolution or oxygen consumption. In order to confirm that the polymers have been completely biodegraded, the amount of generated biomass and other metabolites should be determined. In this study, however, the biomass was not determined because no reliable biomass determination method suitable for carbon balance calculations was available.

CONCLUSIONS

Basic information about the structure of the polymers to be degraded and the enzymes involved in their hydrolysis is required when the biodegradability of polymers by enzymatic hydrolysis is studied. Enzymatic tests can be used with care when fast preliminary information about the biodegradation is needed.

Efficient enzymatic hydrolysis of cellulose requires all the major components of the hydrolytic enzyme system. The right balance between these components was shown to be essential, and especially, sufficient dosages of β-glucosidase were required for rapid enzymatic degradation. It was possible to estimate the degradability using a completely characterized protein solution composed of pure cellulases. However, the culture filtrate was more efficient, probably because it also contained additional hydrolytic enzymes that degrade, e.g., the xylan or glucomannan which are present in wood-derived technical cellulose materials (e.g., paper). In general, the results of the enzymatic hydrolysis and the Sturm test were in good agreement in the case of all the samples, except for cotton fabric. This sample of highly crystalline cellulose was not easily hydrolyzed using enzymes, although it was rapidly decomposed by sludge microorganisms in the Sturm test. Rapid estimation of biodegradability can be carried out by means of a straightforward hydrolysis experiment in 6 h. However, the limitations of using purified enzymes or even culture filtrates in hydrolytic studies has to be considered, although such studies may give important information about the biodegradation potential of the cellulose-based materials. A fast hydrolysis result is strongly dependent on the structural composition of the substrate and is thus best used for comparison of similar types of chemically rather well-defined materials. To obtain a more complete degree of hydrolysis, higher enzyme doses or longer hydrolysis times should be used.

The enzymatic biodegradability of starch-based polymers using enzymes such as glucoamylase and α-amylase is a fast and easy method for obtaining preliminary information about the potential biodegradability of polymers [14]. As shown in this study, a viable sewage sludge microbial population may excrete enzymes that are not present in, e.g., *Trichoderma reesei* or *Aspergillus niger* culture solutions. Therefore, enzymatic tests can be

used solely for preliminary studies. However, if a negative response is obtained, the result should be confirmed with standard tests based on microbiological degradation, using appropriate populations derived from nature or from sewage treatment plants.

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