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Method for the Analysis of Grafted Cellulosic Materials

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Bioplastics is a rapidly emerging topic, to meet technical constraints they, however, often required substantial modifications. The modification of grafting of natural polymers, such as cellulose, is one of the most promising ways to get reliable bioplastics to the market. For the better control of properties and further optimized design of these materials the characterization of grafts is mandatory. This, however, can not be achieved *via* traditional methods, due to different reactivity of natural and synthetic polymers.
An assay based on a catalytic specificity and robustness of cellulolytic enzymes was tested for the selective hydrolysis of the cellulosic matrix of a withdrawn sample and the recovery of the grafts in the organic phase. Among three tested commercial cellulases, *A. niger* cellulase is the most efficient in a biphasic media containing up to 75 % of chloroform. Thus, the sample containing up to 40 g/L of cellulose-based material was transformed in 4 hours by 10 g/L overall enzyme, with no damage for the polyester grafts.
A novel, specific, easy to manage and robust assay for the characterization of grafted cellulosic materials is reported in this research report. Chemically weaker, the ester bond of the polyester grafts is, however, preserved, whereas the ether cellulosic bond is specifically cut by the enzyme.

1. Introduction

Nowadays, cellulases are considered with great interest by the Industrial Biotechnology for either ethanol production (Lin, 2006), conversion of lignocellulosic biomass (Kumar, 2008) or other processes (Percival, 2006). These enzymes are easily produced at industrial scale, even from wastes (Damato, 2010), and offer a promising route to second generation biofuels (Eklund, 1995; Woodward, 1991) and other valuable chemicals such as pyruvic acid, dihydroxyacetone, lactic acid etc. (Lin, 1985). Thus, several aspects of the reactivity of these enzymes were extensively studied, such as catalytic activity (Catellanos, 1995a; Duff, 1986), suitable substrates such as small chromogenic substances (Clayessens, 1992), waste paper (Walpot, 1986), crystalline (Yoon, 2005) or other cellulosic materials (Walker, 1990; Castellanos, 1995b); reproducibility (Esterbauer, 1992) and optimal reaction conditions – size of particles (Peters, 1991), pH and/or temperature (Schulein, 1997, Sreenath, 1993, Cannella, 2010). However, until now only little work has been reported on the activity of cellulases in non-aqueous media such as toluene (Woodward, 1996), chloroform (Schimel, 2006), ionic liquids (Kilpeläinen, 2007) or surfactants (Chen, 2006).
On the other hand, grafted cellulosic polymers become very popular bioplastics for many different applications such as reinforcing fillers (Habibi, 2008; Lonnberg, 2006), biocompatible nanomaterials (Hafren, 2005). Polyesters are usually used as grafts for these materials (Habibi, 2008; Lonnberg, 2006). One of the major problems for the synthesis and characterization of these novel materials is the understanding and the analysis of what is actually grafted. This analysis can hardly be achieved by traditional polymeric techniques such as gel permeation chromatography (GPC) or nuclear magnetic resonance (NMR) as cellulose is not soluble in common solvents. The specific hydrolysis of the cellulosic matrix of a withdrawn sample and the consecutive analysis of the grafts can be a valuable solution for the determination of their type, length and diversity. Unfortunately, chemical hydrolysis cannot be applied in this case, as the ester bond of grafts is weaker than the ether cellulosic bond.
In nowadays quickly evolving world, the innovation often lies at the border of different sciences. Thus, material science techniques appear to be very useful for the monitoring of fermentations (Talon, 2011), as well as the organic chemistry to the industrial biotechnology (Gupta, 2007). In this study, biocatalytic tools

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were applied for the analysis of grafted cellulosic materials. The enzymatic activity of three different commercial cellulases produced by *A. niger*, *Aspergillus sp*. and *Trichoderma viride* in various biphasic conditions was evaluated in order to separate cellulose from grafted organic polyesters. This allows the release and recovery of grafts in the organic phase, which were further analyzed by traditional techniques.

2. Materials and Methods

2.1 Materials

All chemicals were from Sigma Aldrich. The specific activities of used cellulases were: 1 U/mg for the cellulase from *A. niger*, 1 U/mg for the cellulase from *A. sp*. and 7.8 U/mg for the cellulase from *T. viride*.

2.2 Cellulose hydrolysis in bi-phasic medium

Samples (10 mg) of grafted cellulose were stirred in 9.9 mL of reaction medium with 100 mg of cellulase. Hydrolyses were performed at room temperature and under magnetic stirring. The reaction medium was composed of a buffer solution containing different amounts of chloroform.

For the experiments at pH 5 a citrate - phosphate buffer was used, for the experiments at pHs 7 and 9 - phosphate buffers were used. All buffers were at 0.1 M.

2.3 Analysis

Cellulose hydrolysis was monitored through glucose concentration analysis performed with YSI 2700 Select instrument calibrated with 10 g/L glucose solution.

NMR analyses were performed with the Bruker 500 MHz instrument.

Polycaprolactone (PCL) evolution was monitored through GPC analysis of the organic layers; performed with Waters 1515 instrument calibrated with polystyrene (PS) standards. Number average molar mass, M_n, values were calculated from PS equivalents following Eq(1), which corresponds to the equations of Mark-Houwink and Benoït adapted to the PCL polymer (Barakat, 1994), where M_{nPS} stands for measured number average mass of PS and M_{nPCL} stands for the calculated number average mass of PCL.

$$M_{nPCL} = 0.259 M_{nPS}^{1.073} \quad (1)$$

All experiments were performed at least twice.

3. Results and Discussion

3.1 Selection of the most suitable cellulase

The aim of this work was to find a rapid, efficient and specific method for allowing the analysis of grafted cellulosic materials. Thus only commercial, cheap and easily accessible through traditional chemical suppliers cellulases were considered. Moreover common polyesters, such as polycaprolactone (PCL), polylactide (PLA) or polyethylene terephtalate (PET), used for cellulose grafting are mostly soluble in chloroform. Thus the study was focused on this solvent, while it has the log P (partition coefficient between water and octanol) of 1.8 and solvents with the log P below 3 are usually considered as unsuitable for the enzymatic activity (Hocknull, 1990; Terrades, 1993; Collins, 1997).

Three commercial cellulases, produced by different fungus, *i.e. Aspergillus niger*, *Trichoderma viride* and *Aspergillus sp* were tested (Figure 1). Two media were first evaluated: buffer (pH 5) and biphasic medium (buffer / chloroform, 50/50 v/v). Among the three tested cellulases the one from *A. niger* appeared to be more efficient in these conditions, the cellulose hydrolysis was completed in 4 hours. The yields with other cellulases were less important: 50 % for the cellulase from *T. viride*, whereas with the cellulase from *Aspergillus sp* almost no free glucose concentration was detected after 7 hours of the reaction time.

The cellulase from *A. niger* was thus selected for the grafted cellulose hydrolysis. Further, different aspects of robustness and specificity were tested for better understanding of the scope and the limitations of this method.

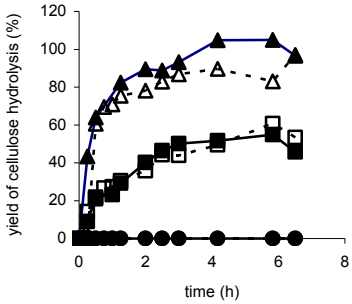

Figure 1: Evaluation of the catalytic activity of the cellulases from A. niger, ∆, A. sp., ◊ and T. viride., □. Bold symbols stand for the experiments performed in the citrate-phosphate buffer alone, empty symbols - for the experiments performed in the medium containing 50 % (v/v) of chloroform.

3.2 Robustness of the method

Robustness towards chloroform concentration

The robustness of the method towards chloroform concentration was studied by submitting cellulose to enzymatic hydrolysis by *A. niger* cellulase in media containing different buffer / chloroform proportions (Figure 2, A). Although in the medium composed by 100 % chloroform the enzyme was no longer active, it showed an impressive stability towards this solvent. Indeed, up to 75 % of chloroform in the reaction medium, no significant loss of hydrolytic activity was observed, and cellulose was quantitatively hydrolyzed in 4 hours, in the way very similar to the hydrolysis in the buffer alone.

Robustness towards pH variations

The robustness of the method towards the pH of the buffer was also evaluated. Thus, alkaline, neutral and acidic conditions were tested for this hydrolysis (Figure 2, B). Significant loss of the activity was observed at neutral and basic pHs, respectively 34.5 % and 84.9 % of the activity drain, comparing to the experiments at pH 5. This result is rather consistent with previous observations on optimal acidic conditions reported for some cellulases (Sreenath, 1993) and cellobiohydrolases (Schulein, 1997). Thus, the weak robustness towards pH variation is a significant limitation of the method.

Robustness towards cellulose concentration

Finally the robustness of the method towards cellulose concentration was also evaluated. Different concentrations of cellulose, ranging from 2 to 40 g/L (Figure 2, C), were tested. The time course of the resulting hydrolysis did not show any significant influence up to 40 g/L of the substrate in the medium.

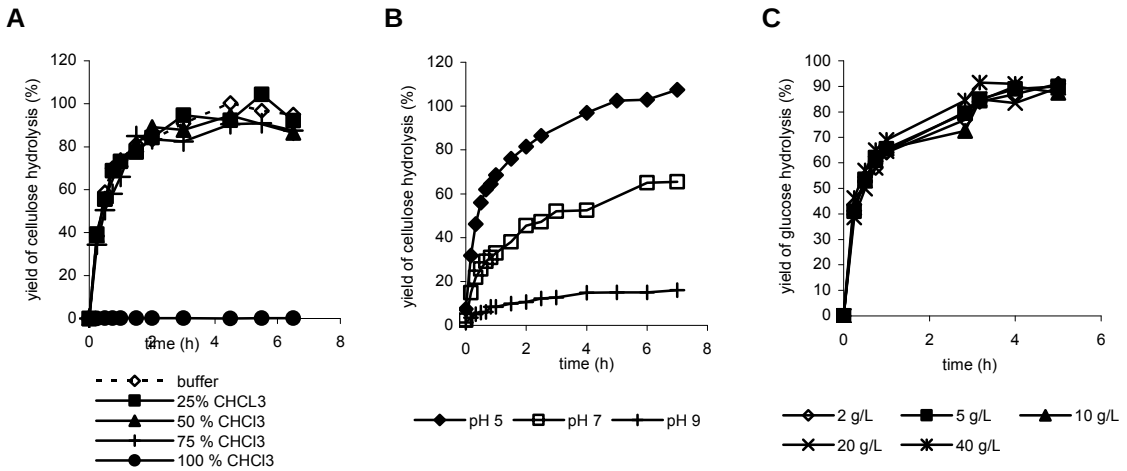

Figure 2: Robustness experiments: evaluation of the influence of chloroform concentration (A), pH shifts (B) and cellulose concentration (C).

Finally, these experiments have demonstrated that the test is robust enough towards chloroform concentration (in the range between 0 and 75 % of the medium) and towards cellulose concentration (in the range between 2 and 40 g/L). However, the test is also very sensitive to the pH of the buffer, which has to be kept as close as possible to 5.

3.3 Specificity of the method

To check the specificity of the method two different criteria were evaluated: the influence of the PCL presence in the reaction medium on the cellulase performance and the conservation of the PCL integrity during cellulose hydrolysis by the *A. niger* cellulase.

The influence of the PCL presence in the reaction medium was evaluated in the range between 0 and 4 g/L (Figure 3, A). The results show that the presence of the PCL did not influence the cellulase activity at any of tested concentrations. Thus this method can be applied even at extremely high (10 %) grafting ratio of cellulose by PCL.

The conservation of the PCL integrity during the cellulose hydrolysis is a crucial point of this method. It was checked by GPC analysis of the organic layers at different PCL concentrations and at different pHs of the buffer solution (Figure 3, B and C). These experiments show that for the variation of PCL concentration between 1.5 and 4 g/L the maximum loss during the reaction process of the number average mass (M_n) is of 1.5 %. The pH of the reaction medium affect more significantly the PCL integrity, in this case the variation can reach 2.2 % at pH 5 and up to 5.5 % at pH 7. Fortunately, the pH 5 appears to be the most suitable for both PCL integrity and enzyme activity (see paragraph above).

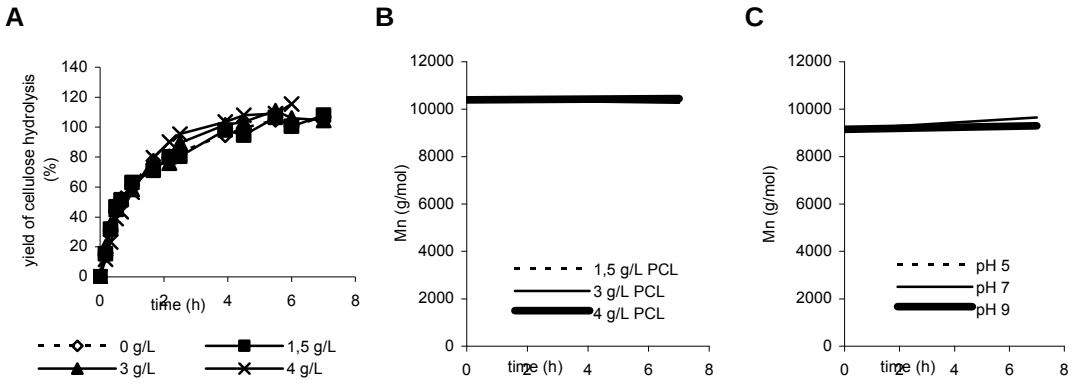

Figure 3: Specificity experiments: evaluation of the influence of different PCL concentration on the hydrolysis time course (A) and on the PCL integrity (B). The evaluation of the influence of different pHs on the PCL integrity (C).

3.4 Validation of the method in the case of cellulose grafted with PCL

In order to confirm the validity of the method, the experiments with 2 different sizes of the PCL grafts were carried out. The analysis performed on the chloroform phase after the cellulose hydrolysis confirmed the presence of PCL. Moreover, as 5 and 10 units oligomers of PCL were used the obtained results showed the convenient ratio of length between the 2 assays and gave important information on the polydispersity of the grafts (Table 1).

These results show both the correct ratio of the different grafts and give complementary information concerning their properties, thus showing the importance of the method for the correct understanding of the features of the obtained grafted materials.

Table 1: Characterization of the PCL grafts of cellulose samples grafted with PCL

PCL used	M_n[1] of grafts	M_p[2] of grafts	Đ[3]
DP[4] 5	467 g/mol	2780 g/mol	5.7
DP[4] 10	946 g/mol	6011 g/mol	5.8

1. number average mass; 2. peak mass; 3. polydispersity; 4. polymerization degree of PCL oligomers

4. Conclusion

An efficient, rapid, robust and specific method for the analysis of grafted cellulosic materials has been described. This method is based on a chemospecific action of *A. niger* cellulase, which selectively destroys cellulosic skeleton from the withdrawn sample material in biphasic medium containing up to 75 % of chloroform. The polymeric grafts are thus recovered in the chloroform phase, ready to use for further characterizations.

The method has demonstrated its robustness towards chloroform and cellulose concentrations. The main weakness of the method relies on the strong dependence of the enzyme activity on the pH of the buffer, which has to be kept at 5.

The chemospecificity of this test was controlled with PCL and can be extended to other types of polyesters. In most suitable test conditions, at pH 5, the grafts are untouched (only 2.2 % of variation of the M_n was observed). This aspect is of crucial importance for the test, as the specificity of the enzyme allow the cellulose hydrolysis with no damage to grafted polyester, whereas in normal, chemical, conditions the ester bond is much weaker than the ether one.

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