Feasibility of Single-Step EDC Coupling for Immobilisation of Xylanase on Biobased Microspheres

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Abstract

The feasibility of the single-step EDC coupling method for immobilisation of xylanase on biobased microspheres, more precisely beads made out of cellulose, was studied. Firstly, a TEMPO (2,2,6,6-tetramethyl-piperdin-1-yl)oxyl oxidation reaction was performed with the goal of producing carboxyl groups (-COOH), on the cellulose beads. Afterwards, the quantity of carboxyl groups was measured by means of conductometric titration. Subsequently. the single-step EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) coupling method was carried out, and the quantity of enzyme coupled to the cellulose beads (post-immobilisation) was determined using the Bradford protein assay. Finally, the catalytic activity of the free enzyme was compared with the catalytic activity of the immobilised enzyme.The catalytic activity of the immobilised enzyme decreased approximately 94 %, relatively to the catalytic activity of the free enzyme. The Bradford protein showed that no more than 0.40 g out of 1 mg of enzyme, attached to the beads. Further experiments indicated the occurrence of cross-linking of the amino groups (-NH_2) and carboxyl groups (-COOH) of xylanase, hampering the desired immobilisation reaction. A way of improving the yield of the single-step EDC coupling is to add the reagents separately (in this experiment, the reagents were added 2h apart from each other) instead of simultaneously. Moreover, more suited methods for the immobilisation of xylanase such as the two-step EDC/Sulfo-NHS coupling method or adsorption techniques should be investigated.

Keywords: Xylanase, enzyme, immobilisation, cellulose beads, oxidation, single-step EDC coupling method.

1. Introduction

Xylanase, an hydrolytic enzyme that cleaves the backbone of xylan, has major applications in the food industry, where it is used to accelerate the baking process, by breaking down hemicellulose. As such, xylanase helps to improve dough features and to increase the quality of bread and other bakery products [1]. Moreover, xylanase can be used on the pharmaceutical industry to produce xylooligosaccharides (XOS), which have health promoting properties that make them suitable for treatment and prevention of gastrointestinal infections, action against skin and hair disorders, osteoperosis, otitis, among others [2]. Enzyme immobilisation allows the reutilisation of the enzyme, leading to lower costs and increased sustainability, and enables an easier separation of the enzyme from the reaction mixture.

Throughout the years, several methods for the immobilisation of enzymes have been developed. These are divided into 4 groups as listed in 1995 by the International Union of Pure and Applied Chemistry (IUPAC): 1) covalent bonding of the enzyme to a water-insoluble matrix; 2) adsorption of the enzyme to a water-insoluble matrix; 3) intermolecular cross-linking of enzyme molecules using multifunctional reagents; 4) entrapment of the enzyme into a water-insoluble matrix or a semipermeable membrane [3]. Covalent bonding has been classified as the type of method that enables the most stable (chemical) interaction between the enzymes and the carriers [4]. An example of a covalent bonding technique is the so-called single-step EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) coupling, where proteins couple to carboxylate particles which uses EDC as the cross-linking agent (figure 1).

EDC is a zero-length crosslinker (i.e. no additional structures such as spacers atoms are added to the formed bond), that covalently links the carboxyl group of one molecule to the amino group of another molecule, forming an amide bond between the two. The first step of the reaction forms

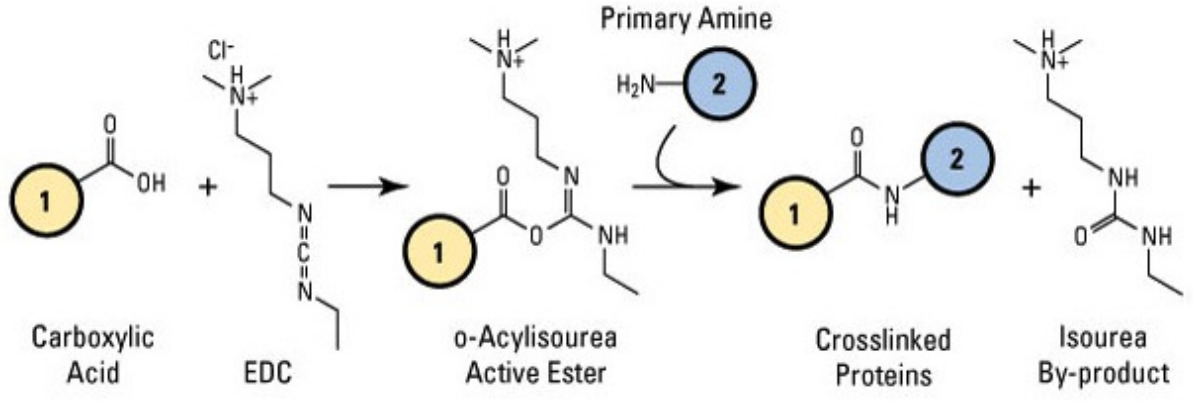

Figure 1: Representation of the single-step EDC coupling reaction steps. (Figure taken from [5]).

an activated *O*-Acylisourea intermediate once the EDC reacts with the carboxyl groups of the cellulose beads. Afterwards, the protein containing the amino group (i.e. xylanase) reacts with the intermediate and forms the amide bond. The desired product is obtained together with a soluble urea derivative, a by-product of the reaction. In the present work, the feasibility of a newly designed method for immobilisation of the xylanase on cellulose beads was studied. The catalytic activity after immobilisation was compared with the catalytic activity of the free enzyme in order to investigate the success of the chosen immobilisation method.

2. Materials & Methods

2.1. Materials

Cellulose beads were provided by the Åbo Akademi University, Turku, Finland. TEMPO 98 % (w/w) (2,2,6,6- tetramethyl-piperdin-1-yl)oxyl was purchased from Sigma-Aldrich. Sodium hypochlorite (NaClO) 10-15 % (w/v), sodium chlorite ($NaClO_2$) 80 % (w/w) and monosodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$) $\geq$ 99 % (w/w) were available in the laboratory. These reagents were used for the TEMPO oxidation. Hydrochloric acid 37 % (HCl) (v/v), sodium hydroxide $\geq$ 98 % (NaOH) (w/w) were purchased from Merck. Sodium chloride $\geq$ 99.5 % (NaCl) (w/w) was purchased from VWR. These were used to perform the conductometric titration. For the single-step EDC coupling, xylanase (endo-1-4-β-Xylanase), X302, was purchased from MetGen Oy, Helsink. EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) $\geq$ 98 % was purchased from Sigma-Aldrich, Trizma base (2-Amino-2-(hydroxymethyl)propane-1,3-diol) $\geq$ 99.9 % (w/w) and di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$) $\geq$ 98 % (w/w) were available in the laboratory. Bovine serum albumin (BSA) protein standard 200 mg/ml and Bradford reagent 0.1-1.4 mg/ml protein required for the Bradford protein assay, were purchased from Sigma-Aldrich. S-AXBL, Azo-xylan from birchwood dyed in Remazolbrilliant Blue R liquid, acquired from Coring System Diagnostix GmbH and ethanol 99.5 % (v/v), available in the laboratory, were used for the assay of endo-1-4-β-xylanase activity.

2.2. TEMPO oxidation of cellulose beads

This experiment was conducted in order to convert the primary hydroxyl groups (-CH_2OH), present on the cellulose beads, into carboxyl groups (-COOH). Four samples (sample 1 to sample 4) of wet cellulose beads of approximately 10 g each were added in 4 different volumetric flasks. To each flask, 100 ml of 50 mM NaH_2PO_4 buffer pH 5.3 was added. The beads and the buffer were left overnight. The next morning, the reagents $NaClO_2$, TEMPO and NaClO were added, in this exact order, to each flask. The quantities of $NaClO_2$ were varied, while the quantities of the remaining reagents were kept constant for all samples (table 1).

Table 1: Amount of reagents used for the TEMPO oxidation (mmol.g^{-1} cellulose beads).

Reagents	Sample 1	Sample 2	Sample 3	Sample 4
$NaClO_2$	1.50	3.10	7.40	12.3
TEMPO	0.600	0.600	0.600	0.600
NaClO	0.200	0.200	0.200	0.200

The oxidation was performed in the water-bath at 60° C for 5 h, at an agitation of 100 rpm. Once the oxidation was finished, the pH of each sample was measured and the beads were washed with ultrapure water Sartorius Stedim until the conductivity

was below 10 µS.cm^{-1} to make sure that there were no residual salts on the beads.

2.3. Fourier Transform Infrared Spectroscopy

Perkin Elmer Spectrum 100 FTIR spectrophotometer was used for spectrometric measurements. This technique allows to detect the presence of the carboxyl groups formed on the cellulose beads by irradiating them with infrared light and by studying the absorption spectra of the samples.

2.4. Conductometric titration

This experiment allows to quantify the amount of carboxyl groups obtained during the TEMPO oxidation, on the cellulose beads. The standard protocol SCAN-CM 65:02 was used [6], with slight changes. After the oxidation, the beads are transformed to proton form, i.e. all the groups receive a positive hydrogen as counter-ion (they are protonised). To do that, the beads were suspended at 1 g.ml^{-1} concentration (i.e. 100 ml of HCl for 1 g of beads) in a 100 mM HCl solution for approximately 30 minutes. The protonisation reaction that took place is as follows: cellulose-COONa + HCl → cellulose-COOH + NaCl. Afterwards, the obtained solution was titrated with a 10 mM NaOH solution at the rate of 0.1 ml.min^{-1}. At each addition of NaOH solution, the conductivity was measured. The titration reaction is the following: Cellulose-COOH + NaOH → Cellulose -COO$^-$ Na$^+$ + H_2O. Sodium chloride was added to the solution in order to improve the accuracy of the conductivity measurements. The beads were freeze dried, before titration, to determine the dry weight that was used in the experiment.

The quantity of carboxyl groups present on the-cellulose beads was calculated according to the used volume of NaOH in phase 2 (figure 2), as illustrated by the following equation: 1.

$$\text{Carboxyl content (mmol.g}^{-1}) = \frac{C \times \Delta V}{m} \qquad (1)$$

where ΔV is equal to V2 minus V1 which represents the used volume (ml) of 10 mM NaOH solution to titrate the existing weak acid groups (-COOH); C is the concentration of the solution of NaOH (mmol.ml^{-1}), and m is the weight of the freeze dried beads (g).

A plot conductivity (µS.cm^{-1}) vs volume of NaOH (ml) similar to figure 2 should be obtained.

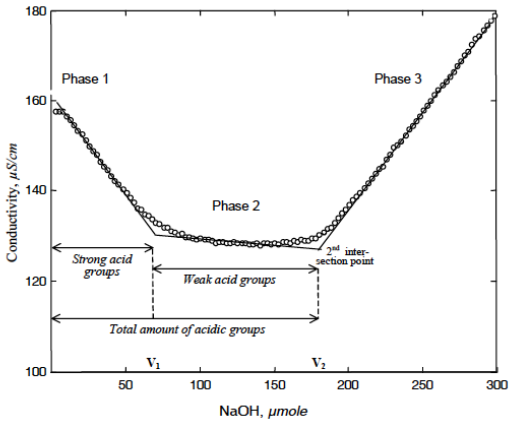

Figure 2: Conductivity (µS.cm^{-1}) vs volume of NaOH (ml) (figure taken from [6]

where phase 1 corresponds to the strong acidic groups, phase 2 corresponds to the weak acidic groups, and the conductivity increase in phase 3 is due to accumulation of NaOH in solution. If there are no strong acidic groups in solution, which is the case, the decrease of conductivity in phase 1 is due to the neutralisation of the protons liberated by the sodium chloride.

2.5. Single-step EDC coupling

This technique is used to attach the enzyme xylanase to the cellulose beads. One buffer solution of 50 mM $Na_2HPO_4.2h_2O$ pH 6.3 was prepared, as well as a buffer of 100 mM Tris-HCl solution pH 6.6. Approximately 1 g of the oxidised cellulose beads from each of the 4 samples (sample 1 to sample 4) were placed in 4 respective tubes of 20 ml (tube 1 to tube 4). The cellulose beads were washed with 5 ml of 50 mM $Na_2HPO_4.2H_2O$ buffer, for three consecutive times. In the meantime, xylanase was dissolved in the same buffer (this solution was designated as E_1). To all four tubes, 5 ml of 50 mM $Na_2HPO_4.2H_2O$ solution and 5 ml of E_1 were added. The amount of EDC added to each tube bears a 1:1 relation with the quantity of carboxyl groups present on the beads. The latter was estimated to be equal to the quantity reported in literature [7] , in lack of the conductometric titration results, which could only be obtained post-hoc. The samples were left reacting for 2 h. Afterwards, the supernatant of each sample was transferred to 20 ml tubes (S_1 to S_4), and the beads were washed three times with a solution of 100 mM Tris-HCl to remove possible remaining (unattached) reagents from the surface.

2.6. Bradford protein assay

The Bradford protein assay is a colorimetric assay that allows to calculate the enzyme concentra-

tion (mg.l^{-1}) in a solution, by means of absorbance reading. The dye, Coomassie Brilliant Blue will bind in acidic pH to the amino acids of the protein, stabilising its anionic form, and allowing to quantify the protein in solution. The supernatants (1 to 4) and the enzyme solution (E1) were tested. All samples were diluted 20-fold since this method only measures below 20 mg.l^{-1}. To each tube, 500 ?l of Coomassie Brilliant Blue G-250 was added.

2.7. Assay of endo-1-4-β-xylanase activity

In order to determine the enzyme activity, before immobilisation and after immobilisation, the protocol of Megazyme [8] was followed. After the single-step EDC coupling method, 0.5 ml of 100 mM $Na_2HPO_4.2H_2O$ solution (buffer) and 0.5 ml of the substrate solution, azo-xylan, were added to the beads. The supernatant (S_1 to S_4) was diluted twofold i.e. 250 µl of the supernatant solution were mixed with 250 µl of the buffer, and the enzyme solution (E_1) was diluted fourfold, i.e. 125 µl of the enzyme solution were mixed with 375 µl of the buffer. To all samples (S_1 to S_4 and E_1), 0.5 ml of azo-xylan was also added. Hence, xylanase depolymerised xylan producing low and high-molecular weight fragments of the original xylan backbone. Subsequently, all prepared samples were placed in the termomixer for 10 minutes, followed by the addition of 2.5 ml of ethanol. Afterwards, the samples were centrifuged (10 minutes) in order to remove the high molecular weight fragments, while the supernatant (low molecular weight fragments) of each sample was poured in a cuvette. The absorbance of the supernatant (i.e. the amount of incident light that is absorbed by the solution) was measured at 590 nm by the DR 5000^{TM} UV-Vis Spectrophotometer. In order to provide a baseline reading for the absorbance it was necessary to prepare, beforehand, a blank solution by adding 2.5 ml of ethanol, 0.5 ml of the substrate solution (azo-xylan) and 0.5 ml of the enzyme solution. The blank solution contains no analyte of interest, and it is used to calibrate the spectrophotometer. The absorbance values were used in the MegaCalc^{TM} provided by the Megazyme website `(www.megazyme.com)` in order to obtain the respective catalytic activities.

3. Results and Discussion

3.1. TEMPO oxidation of cellulose beads

The qualitative results of this experiment indicate that the oxidation was successful. According to Trivedi et al. [7], the colour of the samples should evolve from a light yellow to a dark brown with the increase of $NaClO_2$ added to each sample, and the pH should remain acidic but with a slight increase from sample 1 to sample 4. Figure 3 shows the colour of the samples obtained after 5h of oxidation at 60°C in the water bath.

Figure 3: Samples 1 to 4.$NaClO_2$ increases from left (i.e. sample 1) to right (i.e sample 4) of the oxidised cellulose beads.

Even though there is a bias between these pH values (i.e. 4.56, 5.05, 5.90 and 5.98, for sample 1 to 4, respectively) and the ones from the literature (i.e. 3.60, 4.30, 4.40 and 4.90, for sample 1 to 4, respectively) a similar upward trend over samples is observed. There was an increase of 21%, 15%, 34% and 22% of sample 1 to 4, respectively, relatively to the reference [7]. The higher values in this work can be explained by the pH of the used buffer, which was more basic compared with the reference (i.e pH of $NaH_2PO_4.2H_2O \simeq 4.6$ and 5.4 of the literature and this study, respectively).

The spectra (figure 4) of the reference cellulose beads (i.e. the ones that were not oxidised) and those of the oxidised cellulose beads (figure 4) were obtained by Fourier Transform Infrared Spectroscopy.

As can be seen in figure 4, there is a clear difference between the spectra of the oxidised and non-oxidised cellulose beads. The infrared spectra show for the oxidised beads, a peak of the COO^- stretching band of the carboxylate (the carboxylic acid salt) at approximately 1600 cm^{-1}, while the infrared spectrum of reference cellulose beads does not. This observation is consistent with the literature [9], which has measured the COO^- stretching band between 1610 -1550 cm^{-1}. The non-oxidised beads show no peak in that region, indicating a clear effect of the oxidation reaction.

3.2. Quantification of the carboxyl groups on cellulose beads

The amount of carboxyl groups generated in the cellulose beads was determined by conductometric titration. The obtained results and the ones reported in the literature are listed in table 2.

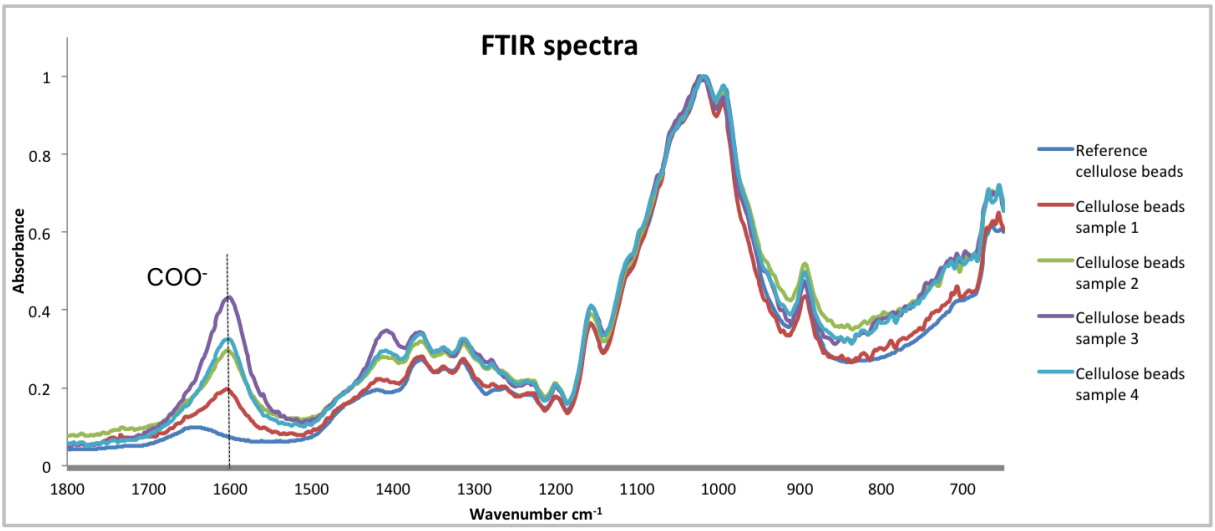

Figure 4: Fourier Transform Infrared Spectra of the reference and oxidised cellulose beads. A characteristic peak of the carboxyl acid salt at 1600 cm^{-1} is evident for samples 1 to 4, which is absent for the reference cellulose beads.

Table 2: Comparison of the quantity of carboxyl groups reported in the literature [7] with this work.

Samples	COOH groups ($mmol.g^{-1}$) reported in the literature [7]	COOH groups ($mmol.g^{-1}$) in the performed experiment
Sample 1	0.560	0.316
Sample 2	0.620	0.426
Sample 3	0.980	0.542
Sample 4	1.35	0.513

The significant difference between the results from literature and the ones in this work are most likely caused by the different structure of the beads. The beads used in the literature were prepared by dropping technique on a small lab scale, while the ones used in this work were freeze-dried, afterwards, on a large scale. This might have led to the production of beads with a less fine-grained resolution and less open pores, hindering the penetration of the reagents.

3.3. Bradford protein assay

The Bradford protein assay was performed to quantify the amount of enzyme that was coupled with the cellulose beads and to correlate this with the catalytic activity explained in the following section. Table 3 presents the amount of xylanase, in mg, that attached to the cellulose beads after the single-step EDC coupling method.

Table 3: Amount, in mg, of xylanase attached to the cellulose beads.

Samples	Mass of xylanase (mg)
Cellulose beads sample 1	0.190
Cellulose beads sample 2	0.234
Cellulose beads sample 3	0.402
Cellulose beads ssample	0.375

A correlation between these results and the catalytic activity is presented in the next section.

3.4. Comparison between the catalytic activity of the free enzyme with the immobilised enzyme

Table 4 presents the mean and standard deviation of catalytic activity which was calculated using the method described in 2.7 obtained for every of the four samples.

Table 4: Catalytic activity (U.mg^{-1} xylanase) of the free and immobilised xylanase.

Samples	Catalytic activity (U.mg^{-1} xylanase)
Free xylanase	13.4 $\pm$3.77
Immobilised xylanase sample 1	0.513 $\pm$0.254
Immobilised xylanase sample 2	0.528 $\pm$0.246
Immobilised xylanase sample 3	0.867 $\pm$0.083
Immobilised xylanase sample 4	0.761 $\pm$ 0.176

These results show a big difference between the catalytic activity of the free enzyme compared with the immobilised enzyme, more precisely a decrease of 97.3 %, 96.2 %, 93.8% and 95% of the catalytic activity of sample 1, 2, 3, and 4, respectively. On the other hand, the obtained values are in accordance with the amount of xylanase attached to the cellulose beads (table 3) since the samples that have a smaller amount of xylanase attached to the cellulose beads, should have a lower catalytic activity such as sample 1 and 2; while the ones with more xylanase attached to the cellulose beads should have a higher catalytic activity.

The immobilisation can be one of the reasons for the obtained catalytic activity results, since it can cause the loss of the enzyme's dynamic properties, alteration of the enzyme's conformation and reduce the access of the substrate to the enzyme's active centre [10]. Some immobilisation processes require the use of carriers. These afect the orientation of the enzyme, which restricts the binding of the substrate or cofactor (if it exists) to the active site [11]. Despite the fact that cellulose beads are considered appropriate carriers for enzyme immobilisation [12], and the reported successful attempts of immobilisation of a wide range of enzymes which show no significant decrease in the catalytic activity [13] [14], with the available data, these hypotheses can not be ruled out, or proven.

3.5. Occurrence of side reactions

The side reactions that EDC can undergo (figure 5) were also taken into account as a possible cause of the unsuccessful immobilisation.

It can be ruled out that the production of *N*-acylisourea derivative is the only probable cause for the failed immobilisation. As explained in section 2.5 the amount of EDC used for the single-step EDC coupling method was determined according to the quantity of carboxyl groups reported in the literature [7]. Therefore, to know if an excess of EDC was used, it was necessary to compare the amount of EDC that was used for the immobilisation technique with the amount of EDC that should have been used. The post-hoc analysis showed that there was an excess of EDC higher than 32 %. If the *N*-acylisourea derivative had been formed due to the excess of EDC, it would render the formation of the amide bond impossible which would lead in the extreme case to a catalytic activity of the immobilised enzyme approximately zero. The results of table 4 show some (low) catalytic activity present after immobilisation. This means that the desired coupling of the carboxyl groups of the cellulose beads with EDC took place (although insufficiently), simultaneously with the undesired *N*-acylisourea derivative formation. Moreover, the cross-linking (explained in the section below) most likely occurred as well, at the same time as these two reactions, and probably at a higher rate.

3.6. Study to detect the occurrence of cross-linking

A simple trial was carried out to prove the occurrence of cross-linking of the amino groups and the carboxyl groups of xylanase. Instead of adding EDC and enzyme solution to the cellulose beads in order to start the immobilisation, solely EDC in varying quantities (i.e. 0.114 g used in sample 1 and 0.266 g used in sample 2) and enzyme solution were added to different tubes. The obtained catalytic activities were 1.38 and 0.710 U.mg^{-1} xylanase. This trial was conducted to determine whether cross-linking took place, which can be detected by a significant decrease of the catalytic activity (since the enzymatic sites of xylanase take part on the cross-linking, and are hence no longer accessible to the substrate). The results show a decrease of more than 90% compared with the catalytic activity of the free enzyme, which implies the occurrence of cross-linking (all parameters where maintained constant between the two conditions except the quantity of EDC). In case the EDC in solution would not interact with the enzyme, the results of the catalytic activity should be equal to the ones of the free enzyme. However, that is not the case. Comparing both samples, it is clear that a higher quantity of EDC is accompanied by a stronger decrease in catalytic activity - the excess of EDC is anti-correlated with catalytic activity. This implies the occurrence of cross-linking, since it seems the only phenomenon capable of explaining this outcome. Since the desired reaction occurs to a much lesser extent than the cross-linking, it can be concluded that the latter reaction (or potentially other competing reactions) is thermodynamically favourable, i.e. has a greater drop in Gibbs energy. However, both the desired and undesired reactions go on simultaneously (al-

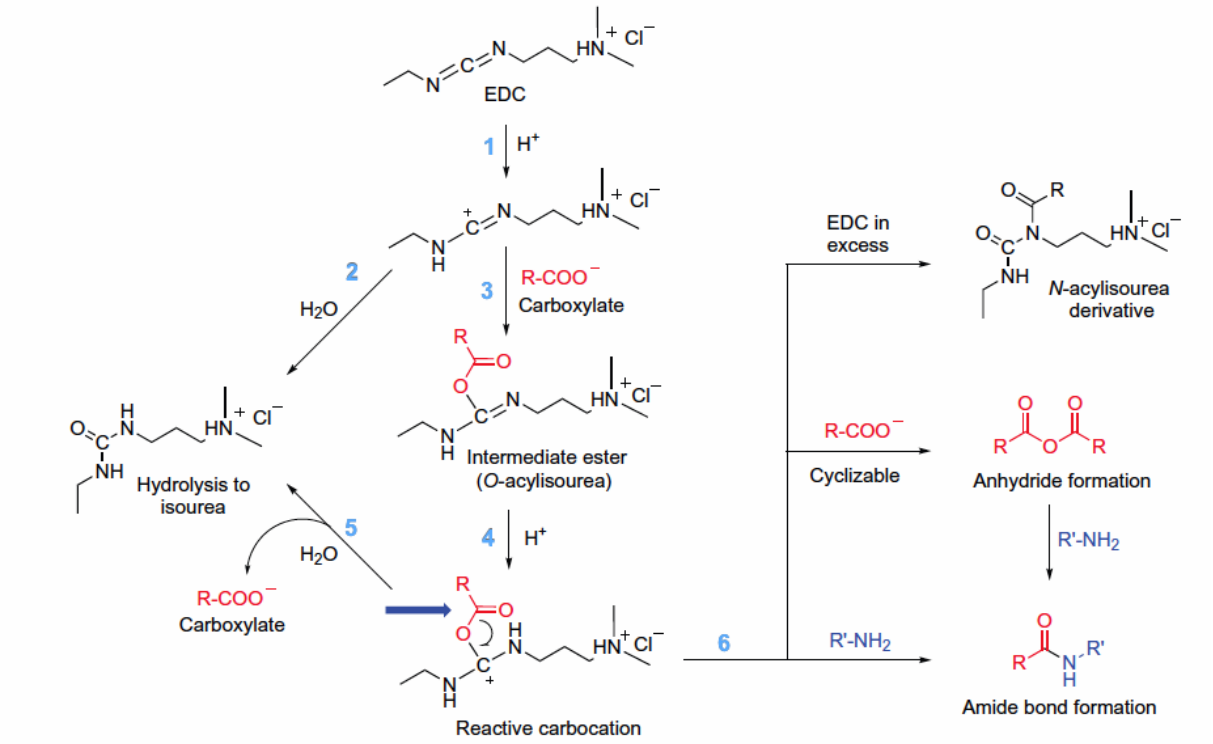

Figure 5: Possible side reactions of EDC that block the formation of the amide bond (figure adapted from [15]).

though at different rates).

3.7. Analysis of the time-frame of the single-step EDC coupling

It seems plausible that the simultaneous addition of EDC and the enzyme solution, could have prevented the coupling of the EDC with the carboxyl groups present on the cellulose beads, and instead allowed the EDC to couple with the ones present on the xylanase, thereby preventing the amide bond formation (between the carrier and the enzyme), and provoking the cross-linking. Therefore, a small adaptation to the followed protocol [15] was made: the EDC was added first and left to react with the cellulose beads for 2h. Afterwards, the enzyme solution was added to the solution and the reaction continued for 2 more hours. The catalytic activity increased more than 28% for samples 2, 3 and 4, but decreased 33% for sample 1. Nonetheless, it is difficult to make a straightforward comparison since the values of obtained for the enzyme activity that followed the protocol were averaged over more (approximately 10) trials while these are not. Hence, the reported activity values of the former are more reliable than the ones of the latter. Moreover, it is unclear why the catalytic activity of sample 2 is so high (i.e. an increase of 440%) compared with the remaining samples.

3.8. Mass-transfer effects

Besides all these parameters discussed above that might have had an influence on the obtained results, it is important to take into account that the yield of the enzyme activity after immobilisation is also dependent on mass-transfer effects [16]. An immobilised enzyme can only catalyse a reaction if the substrate reaches the catalytic sites of the enzyme by diffusion through solution [17]. Afterwards, the formed products diffuse to the bulk solution. Thus, the combination of all these factors lead in practice to a lower substrate conversion rate when using immobilised enzymes than when using free enzymes. In order to avoid diffusional limitations and increase the catalytic efficiency, some measures should be taken such as the use of smaller carriers (promoting easy diffusion, and a large surface-to-volume ratio to maximise the access to the enzyme molecules), and perform the binding of the substrate on the outer shell of the carrier.

4. Conclusions

The feasibility of the single-step EDC coupling method of xylanase on cellulose beads, which was the main goal of this thesis, has proven to be limited. Despite all the addressed arguments, the main reason for the low catalytic activities seems to be the fact that xylanase has both carboxyl and amino

groups in its composition. It seems that the EDC coupled with the carboxyl groups of xylanase (instead of the carboxyl groups of the cellulose beads) forming the *O*-acylisourea, which in turn coupled with the amino groups of xylanase. The crosslinking of xylanase likely prevented the production of the amide bond between xylanase and the carboxyl groups present on the cellulose beads. It seems that this latter (desired) coupling still occurred, although in very low quantities as demonstrated by the results obtained in table 4. More appropriate methods for immobilisation of xylanase must be investigated in future studies. The approach of section 3.7 is something interesting to dig into, and find out if it is productive. For instance, a good candidate might be the two-step EDC/sulfo-NHS coupling method. The addition of the sulfo-NHS to the reaction medium allows the formation of a more stable and active intermediate, as well as the removal of the excess of EDC before adding xylanase, contributing to an increase of the yield and efficiency of the reaction [15] [18]. As mentioned in section 1 other type of methods can be used for the same purpose. The physical methods would be a suitable alternative, since they retain the catalytic activity of the enzymes, especially the adsorption technique, which seems the easiest and promising alternative, offering the best trade-off between process complexity and yield [4].

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