ENZYMATIC PRODUCTION OF GLUCOSE FROM WASTE PAPER

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Municipal wastes have become a severe problem in developed and developing countries during the last century, paper being the main constituent. Not all of the waste paper can be recycled, and therefore alternative procedures for the use of the remaining material should be pursued. The aim of the present work was the application of cellulases for waste paper treatment and the subsequent glucose production and optimization of the conditions for such treatment. Glucose thereafter can be utilized for production of ethanol or other chemicals by specific microbial cultures. The work focused in the stabilization of cellulases by cross-linking or by the addition of specific crown ether based compounds to improve glucose production. The results indicated that enzymatic treatment of waste paper is of particular interest, since it may be an alternative way to carry out municipal wastes treatment and concomitant glucose production. By the application of the proposed procedure, the total amounts of municipal wastes can be greatly reduced and production of bioethanol can be achieved.

Keywords: *Cellulases; Endocellulase; Glucosidase; Cross-linking agents; Crown ethers; Waste treatment; Bioethanol.*

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INTRODUCTION

Municipal wastes have become a severe problem in developed and developing countries during the last century. Among these are the various solid wastes from food, packing, commercial materials, etc.; however, the main constituent of municipal wastes is paper of various types. Recycling of wastes depends on the value of the final product, and it has been applied to the greatest extent for most for metallic materials, i.e. 60% of copper wastes are recycled. Waste paper is recycled at a smaller rate, suggesting that a significant part of municipal waste paper is not useful as recycled products or cannot be recycled due to destruction after its use. It is, therefore, required to develop an efficient way to dispose of such a tremendous amount of waste paper.

The major constituent of paper is cellulose, a homopolysaccharide made up from β-D-glucose. Waste paper, therefore, could be used for glucose production after enzymatic hydrolysis of cellulose by the use of cellulases. Efficient saccharification of cellulose is of great importance from a viewpoint of the disposal of cellulosic wastes and the utilization of cellulose as a renewable resource in relation to the preventing of a greenhouse effect due to carbon dioxide. The enzymatic hydrolysis of waste paper is desirable from the standpoint of green and clean processing, although the process

presents such challenges as a slow reaction rate and low process efficiency, mainly due to the high crystallinity of cellulose, the presence of some lignin, low specific surface area of the material, and the complexity of cellulases as multicomponent enzyme systems, etc.

Cellulases are collectively named as a class of variable multienzyme complexes (van Wyk 1999). They are produced as soluble enzymes, which are diffused from the cells, or are organized as cellulosomes. The soluble enzymes are: I) β-1,4-endoglucanase or endocellulase that cleaves internal β-1,4 glycosidic bonds; II) cellobiohydrolase or exocellulase that removes cellobiose from the non-reducing terminal of cellulose oligosaccharides; and III) β-glucosidase that hydrolyzes cellobiose to β-D-glucose. All three activities act synergistically. The key enzyme seems to be endocellulase, which is responsible for the initial solubilization of cellulose to provide a substrate for the action of both of the other enzymes. Its active site possesses enough space to hold a hexasaccharide, on a glycosidic bond, toward which the catalytic activity is directed. The enzyme acts at moderately low pH, such as 4.5 – 5.0, with the participation of the side carboxylate functions of two acidic amino acid residues (Rouvinen et al. 1990; Divne et al. 1994).

Cellulases belong to a class of unstable enzymes. This is more characteristic in the case of endocellulase. The enzyme retains its initial activity for a limited time at 55 ^{o}C, which is the optimum temperature for activity. It is well known that at such increased temperatures deterioration of polar bonds stabilizing protein structure occur, resulting in unfolding of protein molecules and loss of biologic functions. This is undesirable in industrial processes, taking into account the total costs of the enzymes. In addition, considering the insolubility of the substrate, it seems unlikely that immobilization of the enzyme would help the overall process. Therefore, many studies have investigated thermostable cellulases from thermophilic microorganisms (Maheshwari et al. 2002) or in alternative procedures for their stabilization, such as specific mutation of the cellulase C gene to contain an increased number of cystine residues (Németh et al. 2002) and immobilization of endocellulase to reversibly soluble polymers (Dourado et al. 2002).

Another way to stabilize cellulases should be their selective cross-linking by the use of either Glutaraldehyde (GH) to bridge side aminogroups or 1-Ethyl-3-(3-Dimethylamino-Propyl)-Carbodiimide (EDAC) to bridge side amino groups with carboxyl groups. Small numbers of cross-links should be induced; otherwise the preparations may lose their enzymatic activity, and in addition insoluble enzyme preparations may be obtained. Both GH and EDAC have been used extensively in the immobilization of enzymes on solids supports without affecting their overall properties. Improvement of enzyme stability might also be obtained by the inclusion in the reaction mixture of specifically designed compounds that complex enzyme side groups. Among the additives that can be proposed, crown ethers (CEs) are the most promising. CEs form a class of organic compounds of macrocyclic polyether structure, made up of variable numbers of ethylene glycol units. They contain at least four oxygen atoms in a crown-type structure of at least twelve atoms in total, the ratio of oxygen to carbon atoms being one to two. Their molecular structure, resembling that of a cone with a hydrophilic internal part and a hydrophobic external part, can be used to entrap variously sized cations in non-polar media, depending on their final structure. Through their ability to complex ammonium cations (CEs) can stabilize enzymatic activity in non-aqueous media

(Tsukube et al. 2001; Van Unen et al. 2002). The most widely applied CE in such studies is that containing six oxygen atoms, namely 18-crown-6, which substantially increased transesterification activity of chymotrypsin in non-aqueous media, the increase being related to the hydrophobicity of the solvent used (Van Unen et al. 2002). It was also effective in increasing the activity of other proteases and also lipases (Mine et al. 2001; Van Unen et al. 2001). The observed effect of 18-crown-6 depended on its concentration used, which also depended on the enzymatic activity studied (Mine et al. 2001; Santos et al. 2001; Van Unen et al. 2002).

The aim of the present work is the examination of the optimum conditions for glucose production from various types of waste paper after its treatment by cellulases, i.e., optimum buffer, pH value, temperature, mass ratio, and pretreatment of wastes. The study also focused in the stabilization of enzymes to prolong enzymatic activity and thus to obtain increased glucose production by suitable cross-linking or by the use of newly synthesized specific crown ether derivatives designed in such a way as to incorporate acidic amino acid residues that are present in the enzymatic active site and participating in enzymatic catalysis.

EXPERIMENTAL

Reagents

The enzymes used throughout the study were an endocellulase (C) and a cellobiase (N), both kindly donated by Novozymes A/S (Bagsværd, Denmark). Both enzyme preparations had a protein concentration of about 50 mg/ml. Various types of waste paper, first boiled in water for 1h to remove water soluble impurities, were used as substrate. All other chemicals (salts, acids, bases, cross-linking agents, organic solvents) used were of the best available grade from international suppliers.

Preparation of Cross-linked Enzymes

Cross-linking of the enzymatic preparations was performed using EDAC or GH as agents. Various molar ratios of either cross-linker to the enzymes were used, ranging from 1:1 to 1:5. Cross-linking was carried out in 5 ml of solution at room temperature. In the case of EDAC, cross-linking was performed overnight in water titrated with HCl to pH 4.5, whereas in the case of GH for 2 h in 0.1 M sodium phosphate buffer of pH 7.0, followed by the addition of 0.1 M glycine to react with the excess of GH.

Preparation of CE Derivatives

The key-intermediates 3 and 5 for the synthesis of the projected CE derivatives were obtained, as the corresponding trifluoroacetate salts, through the initial condensation of the commercially available 7,16-diaza-18-crown-6 (1) with the isolable succinimidyl N-trityl-β-alaninate (Tsiakopoulos et al. 2002) to give the bisamide 2. Routine detritylation of 2 gave bisamide 3, whereas $LiAlH_4$-mediated reduction of 2 gave the protected polyamine 4, which upon detritylation gave 'crowned' polyamine 5 (Fig. 1).

Compounds 3 and 5 were bisacylated with Trt-Asp(OBn)-OH (Barlos et al. 1982; Barlos et al. 1984), in the presence of the coupling agent HBTU, or the isolable active

ester TrtNH-Glu(OBn)-OBt, readily obtained from the commercially available H-Glu(OBn)-OH through tritylation followed by activation with HOBt and DCC, to give the polyamides 6a-c.

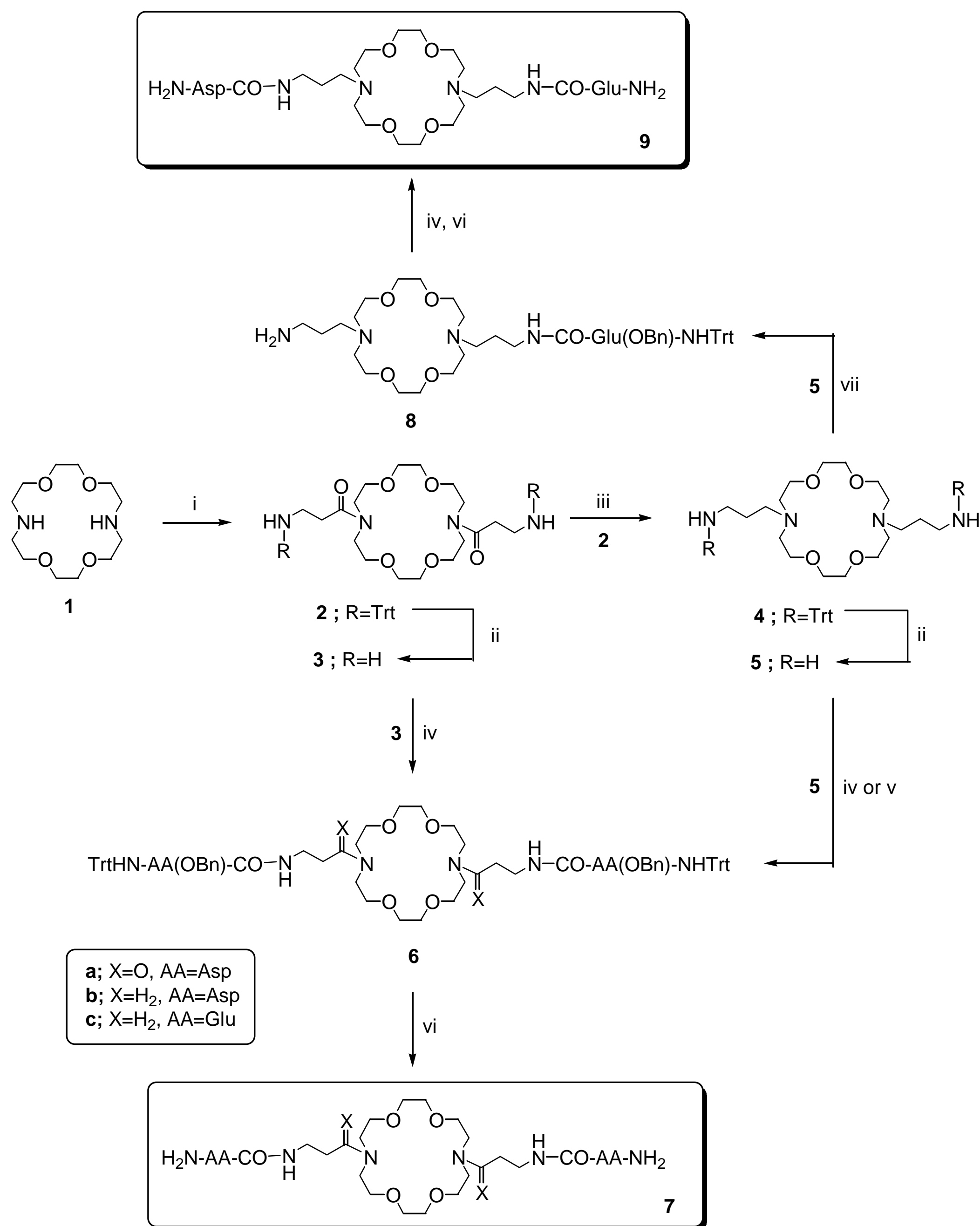

Figure 1: Outline of syntheses for the new CE derivatives described in the present work. Reagents: (i)$TrtNH(CH_2)_2CO_2Su$, Et_3N, DMF/CH_2Cl_2, 65%; (ii) CF_3CO_2H/CH_2Cl_2 (1:3), 90%; (iii) $LiAlH_4$, HF, 65%; (iv) Trt-L-Asp(OBn)-OH, HBTU, iPr_2NEt, DMF, 55-70%; (v) Trt-L-Glu(OBn)-OBt/5 (2:1), iPr2Net, DMF, 75%; (vi) H_2/10% Pd-C, MeOH/EtOAc(1:1) then CF_3CO_2H/CH_2Cl_2 (1:3), 75-90%; (vii) Trt-L-Glu(OBn)-OBt/5 (1:1), $^{i}Pr_2Net$, DMF then FCC, 56%.

TrtNH-Glu(OBn)-OBt, readily obtained from the commercially available H-Glu(OBn)-OH through tritylation followed by activation with HOBt and DCC, to give the polyamides 6a-c. Removal of Bn- and Trt-protecting groups by catalytic hydrogenolysis and acidolysis, respectively, afforded the symmetrical CE derivatives 7a-c, as the corresponding di- or tetratrifluoroacetate salts. On the other hand, selective monoacylation of 5 with TrtNH-Glu(OBn)-OBt, gave the monoamide 8 as the main product, following flash column chromatography purification. Acylation of the remaining primary amino function with Trt-Asp(OBn)-OH and HBTU, followed by complete deprotection, afforded the unsymmetrical CE derivative 9, as the corresponding tetratrifluoroacetate salt.

Cellulase Treatment of Waste Paper

The experiments were performed in a thermo-stable, double-walled batch reactor of 50 ml volume with magnetic stirring. Typically, 400 mg of waste paper were added to the reactor containing 20 ml of buffer and the appropriate enzymes, C (16 μl) and of N (4 μl), as described by the manufacturer (Filos et al. 2006). Incubation at 30 - 75 ^{o}C was maintained for 2-120 h, depending to the experiment, and 0.05 ml were taken for analysis of glucose produced. Glucose was then determined enzymatically by using a commercial kit (Biosystems).

When the effect of CE derivatives on glucose liberation from waste paper had to be determined, their amount was added from a dense stock solution in water.

In some cases, waste paper was subjected to pretreatment for 24 h at room temperature with 0.15 M NaOH or 0.01 M HCl. Pretreatment was also performed by cooling in liquid nitrogen for 15 min and subsequent heating at 250 ^{o}C for 15 min.

Determination of Enzymatic Units and Examination of Cellulases Stability

Protein amounts alone do not give essential information in enzymatic studies and applications. Especially in cases where stabilization of enzymes under specific conditions has to be examined, the determination of catalytic activity is required. Therefore, the enzymatic units of each one of the preparations in either native or cross-linked form, or in the presence of additives was determined, as follows.

The enzymatic units of β-D-glucosidase were determined from a reference curve of p-nitrophenol, after the incubation of native or used enzymatic preparations with p-nitrophenyl-β-D-glucoside for 10 min at 37 ^{o}C, stopping the reaction with four volumes of 0.1 M NaOH and measurement of the absorbance at 400 nm.

The analysis of endocellulase units was performed zymographically (Beguin 1983) in native and cross-linked enzymatic preparations after being incubated for various time periods at increased temperatures. Using this procedure, the endocellulase is well-separated from other (possibly present) enzymes with cellulolytic activities, thus the activity measured is due absolutely to endocellulase. The samples were electrophoresed in 8% polyacrylamide gel and then the gel was placed at the top of an agar sheet containing 0.1% (w/v) carboxymethyl-cellulose. After incubation for 3 h, the polyacrylamide gel was removed and the agar sheet was stained with 0.1% (w/v) congo red for 30 min. The agar sheet was finally destained by 1M NaCl, until white bands were

detected in a red background. The sheet was then subjected to digital scanning, and the enzyme bands were quantified by the Scion PC program.

RESULTS AND DISCUSSION

Treatment of waste paper at 900° C showed that the organic matter content of the towel paper and recycled paper was more than 99% by weight, and that of the copy paper 89%. The amount of cellulose content of wastes that could be solubilized as glucose was identified after their treatment for 120h at 55 ^{o}C with a mixture of C and N in 100-fold excess to that suggested by the manufacturer and measurement of the glucose produced. The result was found to be 72%, 66%, and 40% for towel, recycled and copy paper, respectively.

Stabilization of Enzymes by Cross-linking

The instability of especially endocellulases at elevated temperatures was the main reason for prolonged incubation time for waste paper or cellulosic material treatment. Therefore, the effect of different cross-linking of the enzymes was examined by measuring the enzymatic units of the preparations after they had been incubated for various times at 55 ^{o}C, which is their proposed optimum temperature.

EDAC was more effective in stabilizing C than GH, whereas N was better stabilized by GH. EDAC at a molar ratio to C of 5:1 seemed to produce the optimum effect, since this preparation retained more than 95% of the initial enzymatic units after 4 h incubation at 55 ^{o}C (Table 1). The stabilization of N by GH was successful at a molar ratio of about 15:1, where 75% of the initial enzymatic activity remained after 4 h incubation at 55 ^{o}C (Table 1). An initial small decrease (less than 5%) of activity was observed in all preparations due to the incubation at room temperature during the cross-linking procedure. Cross-linking of C with GH resulted in total loss of enzymatic activity within the first two hours, at every concentration of GH examined, whereas EDAC had no effect on N activity (not shown).

Table 1: C and N Enzymatic Activities (% of initial) after Cross-linking with EDAC and GH, respectively, at Various Molar Ratios, Incubation at 55 ^{o}C, and Determination of the Units of the Preparations.

EDAC/C (molar ratio)	Preincubation time (h)			GH/N (molar ratio)	Preincubation time (h)		
	2	4	24		2	4	24
no EDAC	79	85	38	no GH	37	10	3
1:1	80	88	40	1:1	46	19	8
2:1	81	90	41	5:1	61	30	22
5:1	89	95	41	10:1	71	62	58
10:1	67	75	37	15:1	100	75	64
25:1	53	48	34	20:1	100	69	55

Optimization of the Conditions for the Enzymatic Treatment

Since optimum stabilization of C was obtained after cross-linking with EDAC (molar ratio, 1:5) and of N with GH (molar ratio, 1:15), a mixture of these preparations was used in the subsequent experiments.

Buffer nature

Various buffers of 0.1 M concentration were examined with the different wastes, and in all cases the pH value was set at 4.5, since cross-linking of cellulases did not affect substantially their optimum pH (not shown). The enzymatic hydrolysis was performed at 55 ^{o}C for 24h. The buffers examined are shown in Fig. 2A. In addition, the effect of very dilute HCl (aqHCl, 6 x10^{-5}M) and double distilled water (DDW) was investigated. It was found that acetate buffer permitted the highest production of glucose by copy paper, but very dilute HCl seemed to be optimum for towel paper and recycled paper (Fig. 2A). In weight basis, after treatment of 400mg waste paper for 24h under the optimum conditions, 58, 136, and 20 mg of glucose were liberated from copy, towel, and recycled paper, respectively.

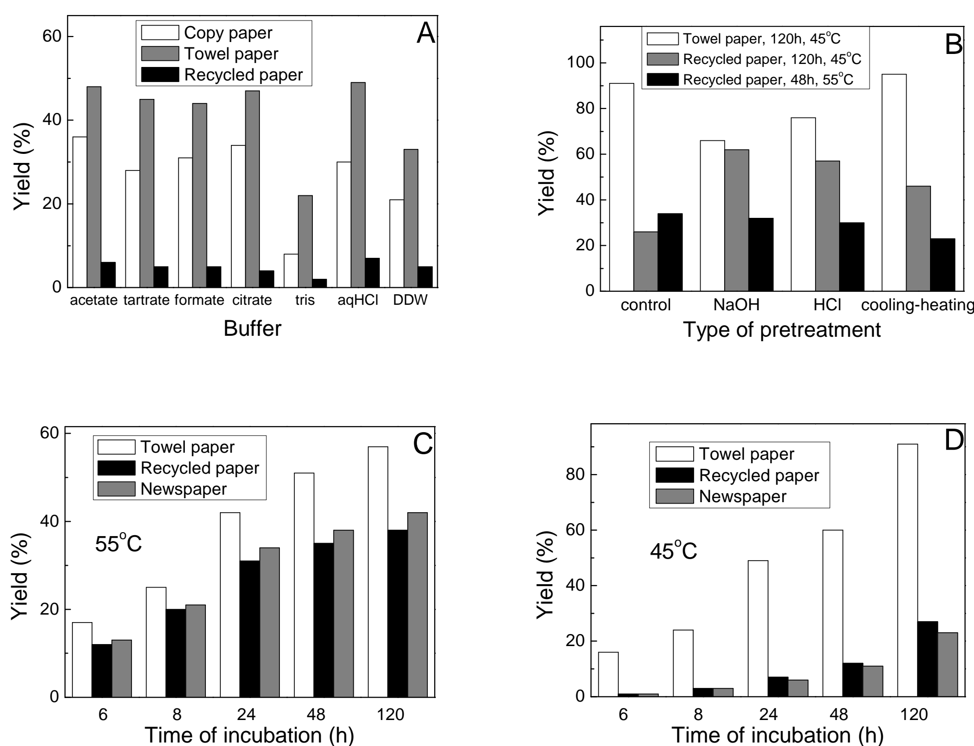

Figure 2: Optimization of conditions for waste paper treatment. A. Buffer. B. Waste paper pretreatment. C and D. Temperature of enzymatic treatment. For additional information, see text.

Waste paper pretreatment

Towel and recycled paper were subjected to various pretreatments to investigate any influence on glucose liberation after the enzymatic treatment. It was observed (Fig. 2B) that pretreatment had different effects in the various wastes. In the case of towel paper a slight decrease in glucose production was observed after pretreatment with NaOH

or HCl, whereas subjection to a cooling-heating process seemed to increase glucose production, but not significantly enough to justify application of this type of pretreatment at an industrial scale. On the other hand, recycled paper was more sensitive to alkali or acid pretreatment. It is noteworthy that pretreatment of recycled paper resulted in increased glucose production, compared to untreated material, only when the subsequent enzymatic hydrolysis was performed at 45 ^{o}C. The liberation of glucose at 45 ^{o}C was about double that of the corresponding test at 55 ^{o}C (160 mg versus 86 mg), and this might be due to the pretreatment that gives to the recycled paper a more soft structure, which permits the action of enzymes continuously but with lower denaturation.

Temperature

Cellulases, in general, are fully active for limited time periods at 50 – 55 ^{o}C. Therefore, the application of these enzymes for prolonged time should take into account the decrease of their activity. To obtain quantitative figures of the ability of the enzymes used under such conditions to degrade cellulosic wastes to glucose, temperatures of 55 and 45 ^{o}C were examined, and glucose production was measured between 6 and 120h (Figs. 2C and D, respectively). The results indicated that the rate of glucose liberation from towel paper was almost constant when the treatment was performed at 45 ^{o}C during the 120h period, resulting in the liberation of about 270 mg of glucose. When the treatment was performed at 55 ^{o}C, the rate of glucose liberation decreased after the first 24h and the amount of liberated glucose after 120h was only 160 mg. Therefore the results suggested that treatment of towel paper was best performed at 45 ^{o}C and for increased time, more than 24 h. On the other hand, treatment of other waste papers, such as newspaper and recycled paper, was best performed at 55 ^{o}C.

Solubilization of waste paper by cross-linked enzymes

Following the above observations, the effect of cross-linked enzymes on glucose production from waste paper was examined. Towel paper was used, since it was very easily solubilized by the enzymes and it also corresponds to non-recyclable wastes. All possible mixtures of enzymes, i.e., cross-linked or not, were used, and treatment of waste paper was performed at 55 ^{o}C for 48 h. This temperature was preferred, since smaller treatment times were required, and the enzymes also retained most of their activity.

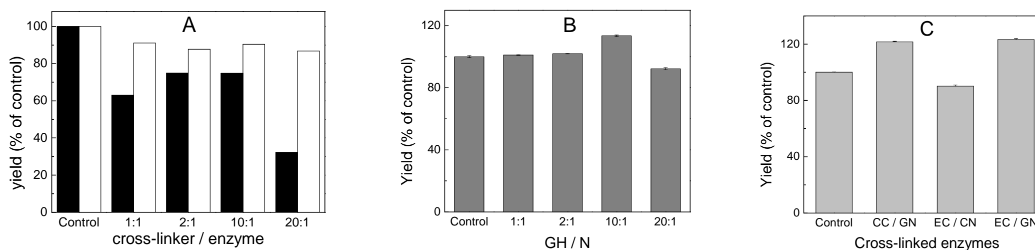

Figure 3: Production of glucose from towel paper at 55 ^{o}C in the presence of cross-linked enzymes, as percentage of that produced after treatment with native enzymes. A. Black boxes: C and N cross-linked with EDAC. White boxes: C and N cross-linked with GH. B. Native C and variously cross-linked N. C. Cross-linked enzymes. CC: Native C. CN: Native N. EC: C cross-linked with EDAC in a molar ratio of 1:5, GN: N cross-linked with GH in a molar ratio of 1:10.

Enzymes cross-linked by using EDAC resulted in decreased glucose production (Fig. 3A, black boxes), whereas those cross-linked by using GH were more effective (Fig. 3A, white boxes). It should be noticed that in the first case, glucose production showed a small peak when the cross-linker was used in the range of 2:1 - 10:1 to the enzyme, which should be attributed to the optimum stabilization of C at a molar ratio to EDAC of about 1:5. In the second case, glucose production was found to be almost constant. Since cross-linking of C with GH highly affected its activity, the results suggested that the high glucose production observed should be attributed to the high activity of cross-linked N.

Therefore, in the subsequent experiment, the enzymes used in the bioreactor were native C and N cross-linked by GH. A slight increase (13%) was observed in glucose production (Fig. 3B) when N was cross-linked with GH in a molar ratio of 1:10. Following this observation, the effect of C cross-linked by EDAC (1:5) mixed by N cross-linked by GH (1:10) in glucose production was examined. The results shown in Fig. 3C suggest that glucose production was increased by 25%, as compared with that produced by control native enzymes.

Effect of crown ether derivatives on waste paper treatment and the stability of cellulases

These series of experiments were performed for 24 h at 55 ^{o}C with towel paper as substrate. The compounds 1, 7a-c and 9 were examined over a wide range of concentrations for their effect on cellulases activity. It was observed that the tetra-amide 7a had the ability to enhance cellulase activity by about 45% over the control system (Fig. 4A), being effective at very low amounts. The underivatized CE 1 did not have any effect within the range of concentrations examined, whereas all other derivatives (7b, c and 9) rather decreased cellulase catalytic activity. The effect of compound 7a on enzymatic activity seemed to reach a plateau at this value. However, by a more detailed measurement of its effect, it was observed that maximum glucose liberation occurred when its molar ratio to cellulase was 10:1 (Fig. 4B), thereafter it decreased and became stable at molar ratios over 100:1. Under such conditions, the plateau value corresponded to 25% increase, compared to the control experiment. It was also observed that this plateau value remained constant up to a molar ratio of 7a to cellulase of 1000:1 (not shown). Compound 7a did not affect optimum temperature and optimum pH value (not shown) of cellulase, when examined at its optimum molar ratio to the enzymes. This observation suggested that this additive did not affect the overall structure of the enzymes or the enzyme-substrate complex. The omission of cellulase in the reaction mixture did not result in the liberation of glucose from waste paper (not shown), suggesting that compound 7a did not posses any catalytic activity. To investigate the ability of compound 7a in enhancing cellulases activity, the compound was mixed with C at a molar ratio of 10:1 and incubated up to two days at 55 ^{o}C. The mixtures were subjected to zymography to determine the endocellulase activity. It was observed (Fig. 4C) that about 95% of the activity was present after 24 h, whereas that of control was less than 40%. Similarly, after 48 h of incubation at 55 ^{o}C, the solutions retained about two-thirds of the enzymatic activity, much more to that observed in control solutions, being about 18 %.

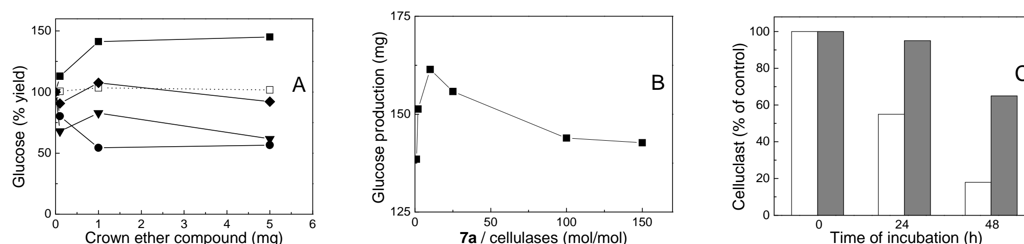

Figure 4: Effect of 7,16-diaza-18-crown-6 derivatives on the activity and stability of cellulases.
A. Various amounts of the analogues or the underivatized CE (1) were added in the bioreactor and the amount of glucose liberated was measured and plotted as percentage of the control (without additive). □: compound 1, ■: compound 7a, ●: compound 7b, ▼: compound 7c, ◆: compound 9.
B. Compound 7a was added in the bioreactor in various molar ratios to the enzymes and the amount of glucose liberated was measured and plotted against molar ratios.
C. C mixed with compound 7a at a molar ratio of 10:1 (black boxes) and C alone (white boxes) were incubated at 55 ^{o}C for various time periods, followed by quantification of the enzymatic activity by zymography.

The results presented herein clearly indicate an effective way to enhance cellulase activity during its application in catalytic processes. Enhancement of the activity of the various cellulases is a general requirement, since this class of enzymes has been extensively studied during processing of agricultural or forest residues (Martin et al., 2002; Soderstrom et al., 2002; Negro et al., 2003; Schell et al., 2003) and byproducts from starch factory (Palmarola-Adrados et al. 2004) for ethanol production. Fuel ethanol is now commercially made mainly from sucrose in sugar cane and from starch in corn kernels and grains (Mielenz 2001; Sun and Cheng 2002). The enzymatic hydrolysis of cellulose is desirable in view of green and clean processing, and a variety of procedures have been proposed for the improvement of cellulase activity, such as transformation of microorganisms (Van Rensburg et al. 1998), selective point mutations (Németh et al. 2002), immobilization (Dourado et al. 2002), or after specific pretreatment of the substrate (Kuo and Lee 2008; Yu et al. 2008). These efforts resulted in effective production of cellulase or production of a more stable enzyme preparation, but to a limited extent. The results of the present study seemed to be superior, since 25% and 45% increases of glucose production were observed by treating towel paper with cross-linked cellulases and with cellulases in the presence of CE derivative **7a**, respectively, suggesting their effective application for at least waste paper treatment, where almost half of the waste weight was solubilized as glucose in only 24 h and under very mild conditions. It should be noted that the increased amounts of liberated glucose were not due to CE derivative activity, as it has been proposed in other cases (Jiang et al. 2005), since the synthesized preparations used did not possess catalytic activity (not shown).

Based on the information obtained from the presented results, it could be proposed that stabilization of cellulases and their application for municipal waste paper treatment and glucose production might be an interesting and applicable industrial process. Waste paper that cannot be recycled, such as towel paper and repeatedly recycled paper, seemed to be excellent substrates for this process and by using cross-linked enzymatic preparations or CEs derivatives the time required could be greatly

decreased. The glucose liberated from these wastes represented 92% and 65% of the total solubilized material, respectively, which corresponded to 70% and 45% of total wastes. Since towel paper and recycled paper should be processed differently, the various types of waste paper should be collected separately. In addition, the use of very dilute HCl solutions in the whole process decreases the cost and facilitates the purification of the products, thus offering environmental benefits.

CONCLUSIONS

1. The use of mixed cross-linked cellulases for waste paper treatment increased glucose production by 25% when applied for 48h.
2. The inclusion of specifically designed additives, such as CE derivatives, showed increased improvement of the net activity of cellulases, which was largely attributed to the stabilization of at least endocellulase activity.
3. The enzymatic treatment of waste paper is of particular interest, since it may be an alternative way for municipal waste paper treatment and concomitant glucose and furthermore alcohol production.
4. Non-recyclable paper can be used to obtain glucose syrups of high purity in glucose that can be subsequently used for commercial or industrial purposes.

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