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Enhanced saccharification of sugarcane bagasse using soluble cellulase supplemented with immobilized β-glucosidase

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HIGHLIGHTS

- β-Glucosidase was immobilized by ionic adsorption and covalent attachment.
- Immobilized β-glucosidase improved by up to 40% the cellulose conversion.
- Immobilized β-glucosidase remained stable up to the third hydrolysis cycle.
- Immobilized β-glucosidase can be an alternative to improve biomass conversion.

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ABSTRACT

The β-glucosidase (BG) enzyme plays a vital role in the hydrolysis of lignocellulosic biomass. Supplementation of the hydrolysis reaction medium with BG can reduce inhibitory effects, leading to greater conversion. In addition, the inclusion of immobilized BG can be a useful way of increasing enzyme stability and recyclability. BG was adsorbed on polyacrylic resin activated by carboxyl groups (BG–PC) and covalently attached to glyoxyl–agarose (BG–GA). BG–PC exhibited similar behavior to soluble BG in the hydrolysis of cellobiose, while BG–GA hydrolyzed the same substrate at a lower rate. However, the thermal stability of BG–GA was higher than that of free BG. Hydrolysis of pretreated sugarcane bagasse catalyzed by soluble cellulase supplemented with immobilized BG improved the conversion by up to 40% after 96 h of reaction. Both derivatives remained stable up to the third cycle and losses of activity were less than 50% after five cycles.

1. Introduction

The enzymatic cocktail required for the bioconversion of biomass into fuels and chemicals is one of the most costly inputs and affects the economic viability of the overall process (Macrelli et al., 2012). In these conversion processes, cellulase enzymes act synergistically in the hydrolysis of cellulose: endoglucanases and exoglucanases or cellobiohydrolase (CBH) act directly on the cellulose fibers, while β-glucosidase (BG) hydrolyzes oligosaccharides and cellobiose to produce glucose (Chundawat et al., 2011; Singhania et al., 2013). Suitable proportions of these enzymes are crucial for an efficient hydrolysis, since the end product of CBH activity (cellobiose) is an inhibitor of the enzymes (Chauve et al., 2010; Lynd et al., 2002). BG activity is therefore important in order to reduce inhibition effects. Furthermore, cellobiose needs to be further degraded to glucose before it can be utilized by conventional yeasts for ethanol production (Liu et al., 2012). Most commercial cellulases are produced by filamentous fungi of the *Trichoderma* genus. However, the amounts of BG secreted by *Trichoderma* are not sufficient for an efficient biomass conversion (Lynd et al., 2002) and commercial preparations usually need to be supplemented with additional BG enzymes. It has recently been reported that BG represents the bottleneck in the overall biomass bioconversion process, due to the fact that it is a key enzyme for complete cellulose hydrolysis (Singhania et al., 2013).

Several studies have aimed to identify a BG with suitable characteristics for biomass conversion, such as thermal stability. Strategies have included cloning and expressing BG genes for enzyme characterization, as well as modification of BG in mutagenesis studies, as recently reviewed by Singhania et al. (2013). Another potential approach for improving enzyme stability is the use of

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immobilization techniques. Enzyme immobilization can increase the rigidity of the protein molecule, with the formation of derivatives that are more stable. Vieira et al. (2011) showed that BG immobilized on an amine/epoxy agarose support was 200 times more stable than the soluble enzyme. Verma et al. (2013) immobilized BG covalently using functionalized nanoparticles and also observed an increase in thermal stability.

It is important to point out that the type of immobilization system directly affects the increase in enzyme stability. Although multipoint covalent binding can result in a derivative with greater stability, immobilization by physical adsorption can enhance the flexibility of the system. Identification of an ideal immobilization system for BG is therefore crucial for achieving the desired characteristics for biomass conversion. There have been few studies that have evaluated the influence of immobilized BG on the efficiency of biomass conversion using lignocellulosic substrates, taking account of enzyme stability and recyclability. Zheng et al. (2013) evaluated the performance of BG immobilized on magnetic chitosan microspheres as a possible recyclable system for the hydrolysis of corn straw. The performance and recyclability of BG immobilized on pretreated spruce was studied by Alftren and Hobley (2013). It was shown that by adding the immobilized BG to free cellulases, the hydrolysis yield of pretreated spruce increased and it was possible to reuse the immobilized BG, with maintenance of enzymatic activity over at least four cycles.

In Brazil, the production of first generation ethanol using sugarcane as feedstock has resulted in the country becoming one of the world's most competitive producers of ethanol (Amorim et al., 2011). The production of second generation ethanol from sugarcane bagasse is an important sustainable means of increasing both yields and the share of renewables in the energy matrix. The development of bioprocesses able to contribute to the viable production of second generation ethanol from sugarcane bagasse is essential. However, to the best of our knowledge, there have been no studies concerning the use of immobilized BG in the hydrolysis of sugarcane bagasse.

This work investigates the immobilization of BG using two different strategies: adsorption on polyacrylic resin activated by carboxyl groups (BG–PC), and multipoint covalent attachment to glyoxyl–agarose (BG–GA). The BG derivatives were then evaluated in terms of their thermal stability, efficiency in the hydrolysis of pretreated sugarcane bagasse, and potential for reuse.

2. Methods

2.1. Materials

Accellerase β-glucosidase (BG) from *Trichoderma reesei* and Accellerase 1500 (cellulase) were gifts from Genencor (Rochester, USA). These enzymes had BG activities around 1156 and 228 U/mL, respectively, measured using 15 mM cellobiose as substrate. Agarose 6B-CL was purchased from GE Healthcare (Uppsala, Sweden), and Immobead D152 (polyacrylic resin functionalized with carboxyl groups) was purchased from ChiralVision (Leiden, The Netherlands). The sugarcane bagasse used in the enzymatic hydrolysis was donated by CTC (Centro de Tecnologia Canavieira, Piracicaba, Brazil).

2.2. Activation of immobilization supports

The glyoxyl–agarose supports were prepared by activating an agarose matrix (6% Sepharose) with glycidol (Sigma, St. Louis) followed by oxidation with periodate (Guisan, 1988). Under gentle agitation and on ice, 105 g of agarose, previously washed with distilled water, was mixed with a cold solution composed of 30 mL of distilled water and 50 mL of 1.7 N NaOH, containing 1.425 g of sodium borohydride. In the next step, 36 mL of glycidol (2,3-epoxy-1-propanol) was added very slowly, to avoid raising the temperature above 25 °C, and the resulting suspension was agitated for 15 h. The etherified material (glyceryl-agarose) was then washed with distilled water under vacuum in a sintered glass filter. After the last washing, the gel was thoroughly dried to remove the interstitial humidity and a 105 g portion was resuspended in 895 mL of water (giving a support to suspension ratio of 1:10), to which 3.21 g of sodium periodate was added. After 2 h of gentle agitation at room temperature, the resulting glyoxyl–agarose support was extensively washed with water and filtered under vacuum.

The polyacrylic resin functionalized with carboxyl groups was initially treated with methanol, washed with distilled water, and dried at 30 °C for 24 h. The dried support was then hydrated with ethanol and washed with distilled water prior to use.

2.3. Chemical modification of the enzyme surface

The surface of the β-glucosidase was chemically modified by inserting amino groups on the Asp and Glu residues. The amination reaction was carried out as described by Tardioli et al. (2011), with some modifications. For this, a BG solution was prepared in 1.0 M ethylenediamine (Nuclear, Brazil) containing 10 mM of N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (Sigma, St. Louis), at pH 4.75, using an enzyme/ethylenediamine solution ratio of 1:10 (v/v). After gently stirring for 2 h, the enzymatic solution was ultra-filtered at 10,000 rpm for 20 min using a 30 kDa cut-off membrane at 4 °C. Finally, the concentrated product was washed three times with deionized water.

2.4. Immobilization of BG on the glyoxyl–agarose support (BG–GA)

The immobilization of BG on glyoxyl–agarose (GA) was performed at 25 °C in 100 mM sodium bicarbonate buffer at pH 9.0 and pH 10.0, using a concentration of 5 mg of protein per gram of gel. A 1 mL aliquot of the reaction medium containing the immobilization buffer and the enzyme was used to measure the initial activity (employed as the control). The immobilization suspension (consisting of the support and the enzyme solution at a ratio of 1:10, w/v) was kept under gentle stirring for periods of 24, 48, 72, and 96 h. The BG activity and the protein concentration in the supernatant were monitored until immobilization equilibrium was achieved. The reaction was terminated by the addition of sodium borohydride to a final concentration of 1 mg/mL and incubation for 30 min at 25 °C. The suspension was then filtered, washed with distilled water, and partially dried under vacuum.

2.5. Immobilization of BG on polyacrylic resin (BG–PC)

After treatment of the resin, 1 g of the support was added to 10 mL of the enzyme solution prepared in 50 mM sodium citrate buffer (pH 4.8) from the commercial enzyme (at 1156 U/mL and 35 $mg_{protein}$/mL). The suspension was kept under gentle stirring at 25 °C until adsorption equilibrium was reached. The BG activity and protein concentration in the supernatant were monitored during the immobilization process. After adsorption equilibrium was reached, the suspension was filtered under vacuum and the immobilized enzyme was washed with distilled water.

2.6. Thermal stability

The thermal stabilities of the immobilized and soluble BG were measured at 65 °C in 50 mM sodium citrate buffer (pH 4.8). The residual activities of the enzyme solution (soluble BG) and the

suspension of immobilized enzyme were measured at regular intervals until the enzyme was completely inactivated. The thermal inactivation model described by Sadana and Henley (1987) was fitted to the experimental data of the relative activity as a function of time in order to estimate the half-life ($t_{1/2}$) of the biocatalyst (the time required for inactivation of 50% of the initial activity). This model is described by

$$A_R = (1 - \alpha) . e^{-k_d . t} + \alpha, \quad (1)$$

where A_R is the relative activity (A/A_0), α is the ratio of the final and initial specific activities, k_d is the first order thermal inactivation constant (h^{-1}), and t is the incubation time of the enzyme solution (h).

The stability factor (SF) was calculated as the ratio between the half-lives of the immobilized and soluble BG, at 65 °C, as described by

$$SF = \frac{t_{1/2,EI}}{t_{1/2,ES}}, \quad (2)$$

where SF is the stability factor, $t_{1/2,ES}$ is the half-life of soluble BG, and $t_{1/2,EI}$ is the half-life of the immobilized BG.

2.7. Stability of soluble BG according to pH

Solutions of soluble BG were incubated at 25 °C using pH values of 4.8 (in 50 mM sodium citrate buffer), 7.0, 8.0, 9.0 (in 50 mM sodium phosphate buffer), and 10.0 (in 100 mM sodium bicarbonate buffer). The residual activities were measured over a period of 24 h.

2.8. Calculation of immobilization parameters

2.8.1. Immobilization yield in terms of protein

The percentage of immobilized protein (IP) was calculated according to

$$IP = \frac{P_0 - P_f}{P_0} \times 100, \quad (3)$$

where IP is the percentage of immobilized protein, P_0 is the protein concentration (mg/mL) in the initial supernatant, which is the concentration of protein in the initial enzyme solution (the immobilization blank), corrected for dilution of the solution due to the water present in the support, and P_f is the protein concentration (mg/mL) measured in the supernatant at the end of the immobilization procedure.

2.8.2. Immobilization yield in terms of activity

The immobilization yield in terms of enzyme activity (IE) was calculated using

$$IE = \frac{U_0 - U_f}{U_0} \times 100, \quad (4)$$

where IE is the percentage immobilization yield, U_0 is the activity of the enzyme solution (immobilization control) measured at the end of the immobilization and expressed in U/g of support (U_0 was measured at the end of the immobilization to account for any enzyme deactivation due to the reaction conditions such as pH, temperature, buffer ionic strength, presence of inhibitors, etc.), and U_f is the activity in the final immobilization supernatant, expressed in U/g of support and corrected for the dilution of the solution due to the water present in the support.

2.8.3. Recovered activity

The recovered activity (RA) was calculated by

$$RA = \frac{U_{EI}}{U_I \times RI} \times 100, \quad (5)$$

where RA is the recovered activity (%), U_{EI} is the apparent activity of the immobilized enzyme (expressed in $U/g_{support}$), and U_I is the real activity available for immobilization (expressed in $U/g_{support}$). U_I was calculated using

$$U_I = \frac{U_{ES} \times V_{ES}}{M_S}, \quad (6)$$

where U_{ES} is the activity of the soluble enzyme (U/mL), V_{ES} is the volume (mL) of enzyme available for immobilization, and M_S is the mass of support (g) used in the immobilization.

2.9. Hydrolysis of cellobiose catalyzed by soluble and immobilized BG

The BG–GA and BG–PC derivatives were evaluated in terms of their efficiency in cellobiose hydrolysis, compared to free soluble BG. Hydrolysis of cellobiose (25 g/L) was performed in 50 mM sodium citrate buffer (pH 4.8) at 50 °C, with constant agitation, for a total period of 72 h. Enzymatic loadings of 56.7 $U/g_{cellobiose}$ were used in all assays. Changes in the reaction over time were monitored by measuring the glucose concentration using a GOD-POD glucose oxidase enzymatic assay kit (Wiener Lab, Rosario, Argentina).

2.10. Hydrolysis of pretreated sugarcane bagasse

Sugarcane bagasse was initially pretreated with a 1% (v/v) H_2SO_4 solution, using a solid to liquid ratio of 1:20. After autoclaving at 120 °C for 30 min, the pulp was treated with 4% (w/v) NaOH, washed with 50 mM citrate buffer (pH 4.8) for pH adjustment, and partially dried under vacuum. The moisture content of the pretreated bagasse was determined before the hydrolysis reactions. Pretreated bagasse (10% w/v, dry basis) was hydrolyzed in 50 mM sodium citrate buffer (pH 4.8) at 50 °C, using soluble cellulase (Accellerase 1500, Genencor) at enzyme loadings of 20 and 40 $FPU/g_{cellulose}$. Hydrolyses were performed under the same conditions after supplementing the reaction medium with immobilized BG (BG–GA and BG–PC derivatives), using an enzyme loading of 120 $U/g_{cellulose}$. The release of glucose was followed during the course of the reaction.

Five batches of pretreated sugarcane bagasse hydrolysis were performed with reuse of the immobilized BG and the residual bagasse. The solid residue (unconverted residue and immobilized BG) remaining at the end of each batch was washed with distilled water and transferred to the next batch containing soluble cellulase and an amount of fresh bagasse sufficient to achieve a final solids ratio of 10% (w/v, dry basis). The glucose released after 24 or 48 h of reaction was quantified using the GOD-POD enzymatic assay kit.

2.11. Protein assays

Protein concentrations were measured using the Bradford method (Bradford, 1976). Serum albumin was used as a protein standard.

2.12. Enzymatic activity assays

The enzymatic activities of the soluble and immobilized BG were determined at 50 °C and pH 4.8 by measuring the initial rate of glucose production using D(+) cellobiose (Sigma–Aldrich, USA) as substrate. Briefly, 1 mL of enzymatic solution (or suspension in the case of the immobilized enzyme) was mixed into 1 mL of 15 mM cellobiose solution prepared using 50 mM sodium citrate buffer at pH 4.8. The reaction was conducted at 50 °C for 15 min, with stirring. The concentration of glucose released was measured using the GOD-POD enzymatic assay kit. The catalytic activity was

expressed as U/mL (where 1 U is equivalent to 1 μmol of glucose released per min, under the reaction conditions).

3. Results and discussion

3.1. Immobilization of BG on the glyoxyl–agarose support

Preliminary tests of BG immobilization on the GA support resulted in low immobilization yields in terms of both protein and enzymatic activity (data not shown). A possible explanation could be that the protein molecule has a low availability of the amino groups required for immobilization. In order to overcome this problem, the surface of the enzyme molecule was chemically modified by carrying out an amination reaction with ethylenediamine (EDA). A high number of amino groups (with pK 9.2) were expected to be inserted into the enzyme molecule, because EDA binds to carboxylic groups and each enzyme molecule contains 59 such groups (Barnett et al., 1991). Furthermore, the chemically inserted amino groups have a pK value lower than that of the amino groups of lysine (pK 10.5), so that the immobilization of aminated BG can be performed using a pH at which the potential for inactivation of the enzyme should be lower. This hypothesis is based on the results of a stability study of soluble commercial and aminated BG in the pH range 4.8–10, which showed that the inactivation profiles were similar in the pH range 4.8–9.0. However, at pH 10, there was almost complete inactivation of the enzyme within the first 24 h (data not shown).

The profiles (Fig. 1a) and yields (Table 1) obtained for the immobilization of aminated BG on GA showed that the duration of the procedure significantly affected the yield. The immobilization reaction time exerted a negative influence on the activity of the enzyme, which could be partially attributed to inactivation at alkaline pH, since the soluble enzyme (control) lost approximately 30% of activity after 72 h, under the reaction conditions (Fig. 1a). The low recovered activity (RA) values of the derivatives (5.1–11.6%) for conditions 1–3 (Table 1) were probably due to the negative effect of the sodium borohydride used in the reduction step of the derivatives (Blanco and Guisan, 1989). In addition, the formation of multiple covalent bonds in the enzyme-support could lead to conformational changes in the tertiary structure of the enzyme molecule, contributing to its inactivation.

An attempt to improve the RA values of the derivatives was carried out by adding glucose (which is a competitive inhibitor) to the reaction medium. The presence of a competitive inhibitor during the immobilization process can help to preserve the activity of the immobilized enzyme, because it prevents the formation of covalent bonds that lead to distortion of the active site of the enzyme (Tardioli et al., 2003). However, it is important to point out that reducing the number of covalent bonds can also lead to a reduction in the stability of the immobilized enzyme. Fig. 1b shows the profile of immobilization of aminated BG onto GA in the presence of a competitive inhibitor (10 mM of glucose). The beneficial effect of glucose can be observed by comparing Fig. 1a and b, from which it can be seen that the immobilization rate was not affected by the presence of the inhibitor, since the profiles of the protein concentration in the supernatant were similar for both immobilization conditions. Furthermore, the presence of glucose (10 mM) during the immobilization process significantly contributed to the preservation of BG activity, as the soluble enzyme remained fully active for 72 h at pH 9.0 (Fig. 1b). The recovery of enzymatic activity in the suspension (enzyme in the supernatant plus immobilized enzyme) was approximately 70% in the presence of glucose, in contrast to only about 35% recovery in the absence of glucose (as reflected in the suspension activities shown in Fig. 1a and b).

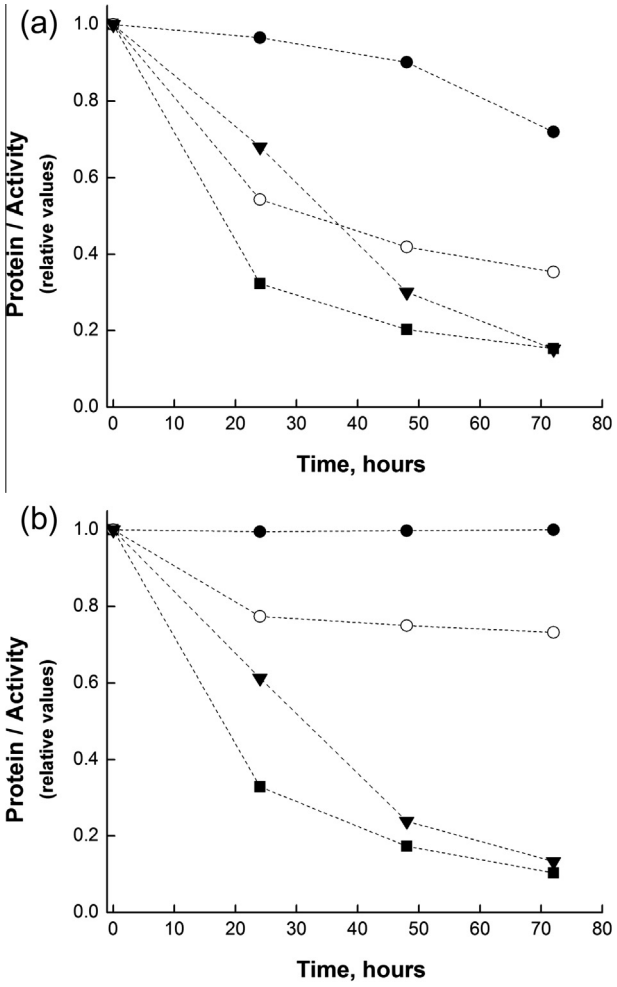

Fig. 1. Immobilization profile of aminated BG on glyoxyl–agarose at 25 °C, pH 9.0 (50 mM sodium bicarbonate buffer), in the absence (a), and presence (b) of 10 mM glucose. Legend: (●) control; (○) suspension activity; (▼) supernatant activity; (■) supernatant protein.

Although the presence of glucose was effective in preserving BG activity during the immobilization process, this benefit was not observed during the reduction step with sodium borohydride. The effect was negligible, even at a glucose concentration five times higher (50 mM) (Table 1). Recovered activity values in the range 12–18% were obtained for the BG–GA derivatives, which supports the notion that the sodium borohydride reaction step had a deleterious effect on BG activity. Borohydride was used in the final step of the immobilization procedure for two purposes: stabilization of the enzyme-support linkages (transformation of Schiff bases into stable secondary amino bonds) and reduction of unreacted aldehyde groups to inert hydroxyl. However, this chemical can have important deleterious effects on protein structures, such as disulfide bond splitting or reductive cleavage of peptide bonds, which can affect the activity and/or stability of the immobilized enzyme (Blanco and Guisan, 1989), as probably occurred here in the case of the BG–GA derivatives.

3.2. Immobilization of BG on polyacrylic resin activated by carboxyl groups (BG–PC)

The immobilization of BG on polyacrylic resin activated by carboxyl groups proceeds by attraction between the negative ionic groups of the support (carboxylic groups) and the positive groups

Table 1
Immobilization yield of the BG–GA derivatives (aminated BG immobilized on glyoxyl–agarose).

Test	Immobilization conditions	IP (%)	U_{IE} (U/$g_{support}$)	RA (%)
1	pH 9.0; 25 °C; 24 h	67.7	3.8	11.6
2	pH 9.0; 25 °C; 48 h	80	3.4	5.6
3	pH 9.0; 25 °C; 72 h	85	2.6	5.1
4	pH 9.0; 25 °C; 24 h; 10 mM glucose	67.1	3.8	11.6
5	pH 9.0; 25 °C; 48 h; 10 mM glucose	83	6.4	14.2
6	pH 9.0; 25 °C; 72 h; 10 mM glucose	90	7.6	12.4
7	pH 9.0; 25 °C; 72 h; 10 mM glucose; reduction with sodium borohydride in the presence of 50 mM glucose	90	9.0	18.2

IP = protein immobilization yield; U_{IE} = apparent activity of the immobilized enzyme.

of the enzyme (side chains of positively charged amino acid residues). It can be seen from Fig. 2 that adsorption equilibrium (in terms of protein and activity) was achieved after 3 h of immobilization reaction. At the end of 4 h, the immobilization yields were 70% and 35% in terms of protein and BG activity, respectively. The prepared BG–PC derivative showed an activity of 17.86 U/$g_{support}$, representing a recovered activity of 97%.

Highly loaded biocatalysts are desirable for industrial purposes because they provide greater catalytic activity per reaction volume, increasing the reaction rate and consequently decreasing the time required to achieve the conversion. High volumetric productivities are reflected in lower process costs. The capacity of the support was therefore evaluated using protein loadings varying from 3.5 to 52.8 $mg_{protein}/g_{support}$. The experimental conditions were 25 °C, pH 4.8, and 24 h (the time required to reach adsorption equilibrium in all assays).

In terms of protein, the immobilization yields exceeded 80% for almost all the loadings tested, and it was possible to load the biocatalyst with up to 44 $mg_{protein}/g_{support}$ (Table 2). This showed that the support possessed a high surface area, indicative of substantial porosity. However, it should be pointed out that a greater presence of large molecules within the pores can decrease the intra-particle diffusion rates of substrates and products. This is a very common phenomenon observed for highly-loaded immobilized enzymes (Tardioli et al., 2005).

3.3. Thermal stability of BG immobilized on the glyoxyl–agarose support

The thermal stabilities of the BG–GA derivatives obtained using different reaction conditions were first evaluated by incubating the

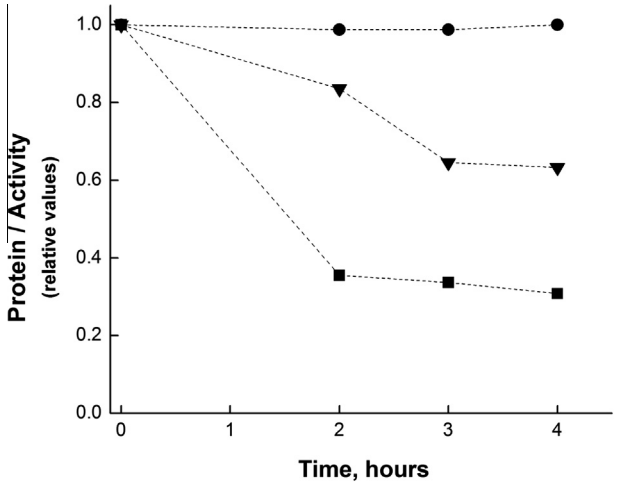

Fig. 2. Immobilization profile of BG on carboxyl polyacrylic resin at 25 °C, pH 4.8 (50 mM sodium citrate buffer). Legend: (●) control; (▼) supernatant activity; (■) supernatant protein.

derivatives at 65 °C in 50 mM sodium citrate buffer (pH 4.8) for 4 h (Fig. 3). Similar inactivation profiles were observed for BG immobilized on GA at pH 10 for 1 h and at pH 9 for 24 h. A likely explanation is that covalent bonds are formed more slowly at lower pH. On the other hand, the derivatives prepared at pH 9 using reaction times of 48 and 72 h were inactivated more slowly, probably due to the formation of multiple linkages between the enzyme and the support, which increased the stability of the derivatives.

In order to estimate the half-life ($t_{1/2}$) of BG immobilized on GA at pH 9 using a reaction time of 72 h, the incubation of the derivative at 65 °C and pH 4.8 was extended to 27 h (insert of Fig. 3). The experimental data shown in Fig. 3 were used to determine the thermal inactivation parameters (Table 3). The thermal deactivation constant (k_d) and half-life ($t_{1/2}$) were calculated using the exponential nonlinear fit method (Sadana and Henley, 1987). It was found that the thermal stability of the BG immobilized on GA at pH 10 for 1 h and at pH 9 for 24 and 48 h was between two and three times higher than that of the soluble BG enzyme. Moreover, the derivative immobilized at pH 9 for 72 h was 19 times more stable than the soluble BG enzyme. The increased stability can be attributed to the formation of multipoint covalent bonds between BG and GA, assisted by the longer contact time between the enzyme and the support, resulting in a more rigid structure of the immobilized molecule (reduced mobility of the protein structure). The BG–GA derivative immobilized at pH 9 for 72 h was therefore selected for further evaluation in the hydrolysis trials. It is important to point that the pH 9 is necessary only for immobilization procedure. The unstable Schiff's bases (CH=N) formed between the amino groups from the enzyme and the aldehyde groups from the support are converted into very stable secondary amine bonds by reduction with sodium borohydride (Blanco and Guisan, 1989). Thus, the biocatalyst BG–GA can be used at 4.8 without leakage of enzyme from the support due to the nature of the very stable covalent linkage established between the enzyme and the support.

Improvement in the thermal stability of BG following immobilization by covalent binding has been reported previously. For example, Verma et al. (2013) observed that *Aspergillus niger* BG immobilized on magnetic nanoparticles activated with glutaraldehyde was approximately 5 times more stable at 70 °C, compared to the soluble enzyme. Singh et al. (2011) showed that the half-life at 65 °C of *Agaricus arvensis* BG covalently immobilized on functionalized silicon oxide nanoparticles was increased 288-fold, compared to the free enzyme. Vieira et al. (2011) found that BG from Novozymes, covalently immobilized on amine-epoxy agarose, was 200 times more stable at 65 °C and pH 4.8, compared to the soluble counterpart. Ahmed et al. (2013) immobilized BG from *A. niger* on sponge activated with glutaraldehyde, but the immobilized enzyme showed no notable improvement in stability, compared to the soluble enzyme. It is therefore clear that the degree of stabilization of an enzyme depends on the microorganism from which it originates, as well as the type of anchorage of the enzyme to the

Table 2
Adsorption of BG on polyacrylic resin functionalized with carboxyl groups (BG–PC), at 25 °C in sodium citrate buffer (50 mM, pH 4.8), after 24 h of reaction.

Protein load ($mg_{protein}/g_{support}$)	Immobilized protein, q_e ($mg_{protein}/g_{support}$)	Immobilization yield, IP (%)
3.5	3.1	89
8.6	6.0	70
20.6	16.5	80
32.2	27.2	84
52.8	44.3	84

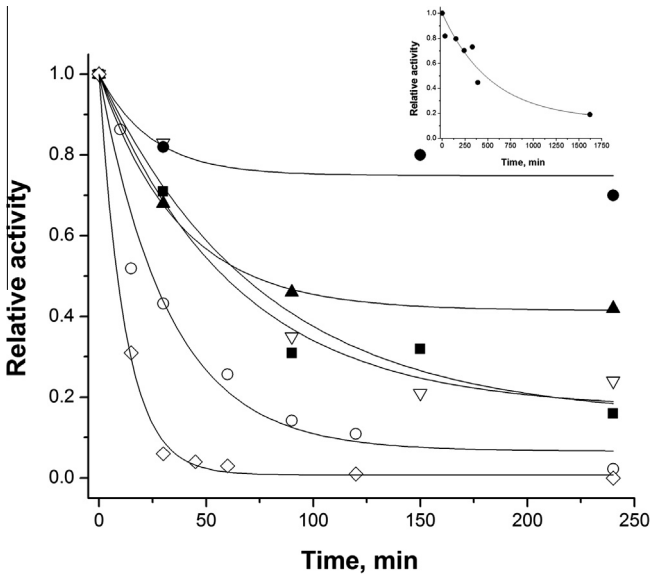

Fig. 3. Profile of thermal inactivation (65 °C, pH 4.8) of free and immobilized BG. Legend: Soluble BG (○); BG immobilized on PC at pH 4.8 for 24 h (◇); BG immobilized on GA at pH 9.0 for 24 h (▽), 48 h (▲), and 72 h (●); BG immobilized on GA at pH 10.0 for 1 h (■).

Table 3
Inactivation parameters of soluble and immobilized BG at 65 °C and pH 4.8 (in 50 mM sodium citrate buffer).

Biocatalyst	k_d (min^{-1})	α_1	$t_{1/2}$ (min)	SF
Soluble BG	0.0308 ± 0.0057	0.0668 ± 0.0530	25.0	1.0
BG–GA; pH 10; 1 h	0.0159 ± 0.0038	0.1714 ± 0.0630	58.2	2.3
BG–GA; pH 9; 24 h	0.0132 ± 0.0048	0.1486 ± 0.1107	67.0	2.7
BG–GA; pH 9; 48 h	0.0269 ± 0.0009	0.4150 ± 0.0058	71.7	2.9
BG–GA; pH 9; 72 h	0.0019 ± 0.0006	0.1465 ± 0.1304	471.4	18.9
BG–PC; pH 4.8; 24 h	0.0825 ± 0.0042	0.0075 ± 0.0094	8.5	0.34

k_d = first order inactivation constant; $t_{1/2}$ = half-life; SF = stabilization factor.

activated support, temperature, and inactivation pH, amongst other factors.

3.4. Thermal stability of BG immobilized on the polyacrylic resin

The stability of the BG–PC derivative was evaluated by incubation in 50 mM citrate buffer (pH 4.8) at 65 °C for 4 h, under the same conditions used for the soluble enzyme. It was found that the immobilized enzyme was inactivated faster than the soluble enzyme (Fig. 3), and that the latter was approximately three times more stable (Table 3). The lower stability of the immobilized enzyme can probably be explained by the fact that the immobilization on PC resin occurred due to adsorption. Immobilization by adsorption does not usually lead to any substantial enzyme stabilization, due to the weak nature of the bonds (such as van der Waals and ionic forces) between the support and the enzyme molecules. This permits rapid desorption of the enzyme from the support, depending on the conditions (pH and ionic strength) under which the biocatalyst is used (Blanch and Clark, 1987). Moreover, the soluble commercial enzyme formulation contains preservatives to increase the shelf-life. These preservatives are removed during immobilization of the enzyme, which could therefore lead to a shorter half-life. Nevertheless, an important advantage of the enzyme immobilized by adsorption is that it is easy to remove the enzyme from the support in order to regenerate the biocatalyst. The inactive adsorbed enzyme can be stripped from the support and replaced with active enzyme, in some cases even without removing the support itself from the reactor (Blanch and Clark, 1987). Depending on the application of the biocatalyst, this factor should be taken into consideration when selecting an immobilization procedure.

3.5. Hydrolysis of commercial cellobiose

The BG–GA and BG–PC derivatives were firstly evaluated in terms of their efficiency in cellobiose hydrolysis, compared to free soluble BG. Commercial cellobiose (25 g/L) was hydrolyzed at 50 °C in 50 mM sodium citrate buffer (pH 4.8) for a total period of 72 h. An enzyme/substrate ratio of 56.7 $U/g_{cellobiose}$ was used in all these experiments. The use of soluble BG resulted in complete conversion of cellobiose to glucose within 12 h of reaction (Fig. 4). Using the BG–PC derivative, the profile was similar to the hydrolysis catalyzed by the soluble enzyme at the beginning of the reaction, reaching 90% conversion after 12 h and up to 97% conversion after 24 h of reaction. However, the BG–GA derivative hydrolyzed cellobiose more slowly, requiring 36 h to reach 89% conversion.

These hydrolysis results were very encouraging; however, from the industrial perspective it is important to determine the biomass conversion performance of immobilized BG using lignocellulosic substrates, considering both stability and recyclability. The next step was therefore to evaluate the hydrolysis of sugarcane bagasse using a cellulase preparation supplemented with the BG–GA and BG–PC derivatives.

3.6. Hydrolysis of sugarcane bagasse

Pretreated sugarcane bagasse (10% w/v, dry basis) was hydrolyzed at 50 °C in 50 mM sodium citrate buffer (pH 4.8) for 24 h using soluble cellulase (Accellerase 1500, Genencor) at an enzyme loading of 20 $FPU/g_{cellulose}$. Hydrolyses were also performed under the same conditions after supplementing the reaction medium with immobilized BG (BG–GA and BG–PC) at an enzyme loading of 120 $U/g_{cellulose}$. The supplementation of the cellulase preparation with immobilized BG (BG–PC and BG–GA derivatives) resulted in conversions of cellulose to glucose approximately twice more high: (22.2 ± 1.7)% for cellulase only, (44.6 ± 4.8)% for cellulase supplemented with BG–PC, and (42.5 ± 4.9)% for cellulase supplemented with BG–GA. This result could be explained by a reduction in the inhibitory effect of cellobiose (Singhania et al., 2013). Most importantly, these results demonstrated that the immobilized BGs were very effective in hydrolyzing a real substrate (sugarcane bagasse).

The performance of the derivatives in the hydrolysis of pretreated sugarcane bagasse was investigated over longer periods of time, using the same conditions described above, except that the cellulase loading was increased to 40 $FPU/g_{cellulose}$. A conversion close to 100% could be obtained within 96 h of reaction when the reaction medium was supplemented with BG–PC (Fig. 5). For this set of experiments, the performance of BG–PC was also superior, compared to that of BG–GA, corroborating the hypothesis that

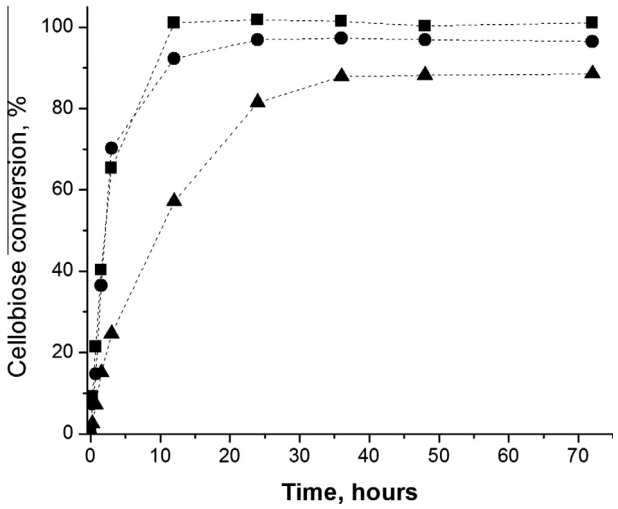

Fig. 4. Cellobiose hydrolyses at 50 °C and pH 4.8, catalyzed by free soluble BG (■) and BG–GA (▲) and BG–PC (●) immobilized derivatives using an enzyme/substrate ratio of 56.7 U/$g_{cellobiose}$.

the covalent linkage of the BG–GA derivative might impair its activity.

In order to evaluate the potential recyclability of the derivatives, the hydrolysis of sugarcane bagasse at 50 °C in 50 mM sodium citrate buffer (pH 4.8) was performed for 24 and 48 h, with reuse of the immobilized BG and residual bagasse. The solid residue (unconverted residue and immobilized BG) remaining at the end of each batch was washed with distilled water and transferred to the next batch containing soluble cellulase and an amount of fresh bagasse sufficient to achieve a final solids ratio of 10% (w/v, dry basis). It was found that the conversion remained very stable up to the third cycle, and then decreased from about 48% or 62% (first batch, Fig. 6a and b, respectively) to 25% or 35% (fifth batch, Fig. 6a and b, respectively).

The results presented here are very promising when compared to other studies reported in the literature. In earlier work, Tu et al. (2006) showed that immobilized BG was stable for up to six cycles of cellulose hydrolysis at 45 °C. Although other studies have

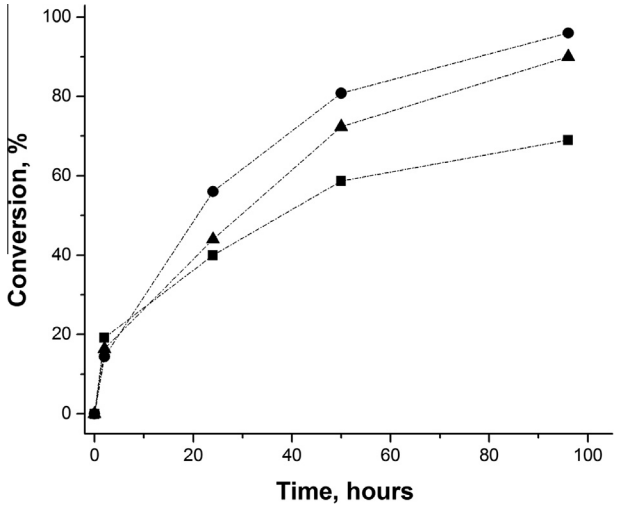

Fig. 5. Sugarcane bagasse hydrolyses at 50 °C and pH 4.8, catalyzed by soluble cellulase (■) and soluble cellulase supplemented with 120 U/$g_{cellulose}$ of BG–PC (●) and (▲) BG–GA. Cellulase load of 40 FPU/$g_{cellulose}$ for all assays. The values are plotted as means ± standard deviations.

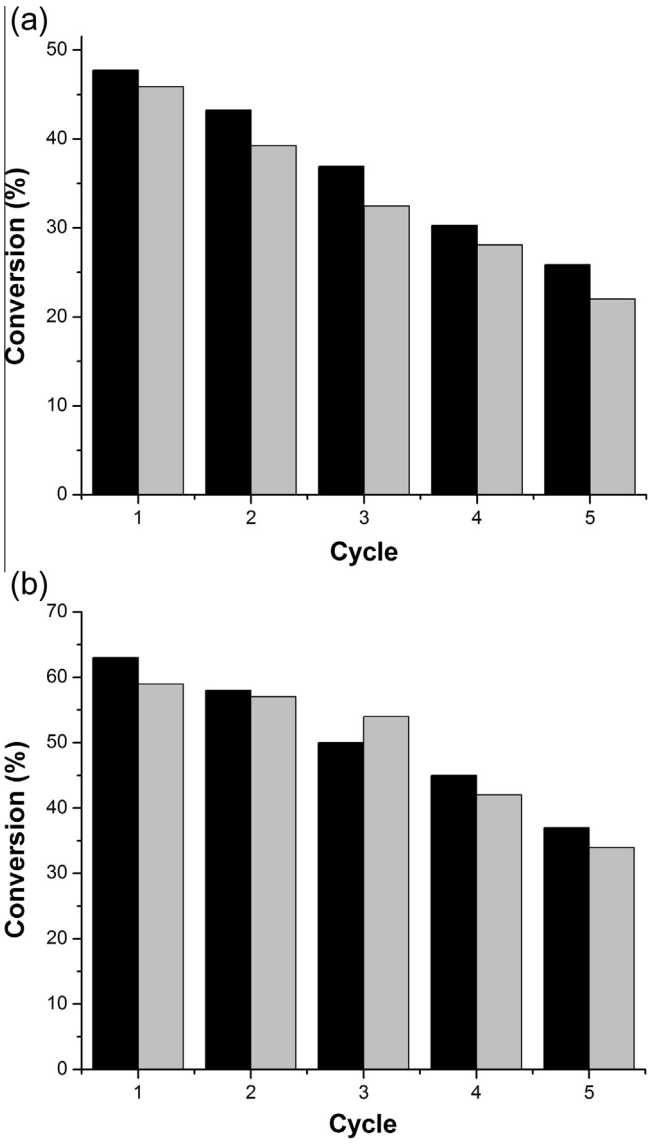

Fig. 6. Repeated hydrolyses of bagasse at 50 °C and pH 4.8, using (a) soluble cellulase (20 FPU/$g_{cellulose}$) for 24 h, and (b) soluble cellulase (60 FPU/$g_{cellulose}$) supplemented with 120 U/$g_{cellulose}$ of BG immobilized on PC (black bars) and GA (gray bars) for 48 h.

reported greater numbers of cycles for BG derivatives, a commercial substrate (either cellobiose or pNPG) was usually used for a shorter period of time (10–15 min) (Singh et al., 2011; Verma et al., 2013) or at a lower reaction temperature (Wang et al., 2009). Therefore, to the best of our knowledge, this is the first study showing the potential of using and recycling immobilized BG to supplement a cellulase cocktail in the hydrolysis of sugarcane bagasse.

4. Conclusions

Immobilization of BG was performed using polyacrylic resin activated by carboxyl groups (PC) and multipoint covalent attachment on glyoxyl–agarose (GA). The thermal stability of BG–GA at 65 °C was up to 19 times greater than that of free BG, while BG–PC showed lower thermal stability than the free commercial preparation. Nevertheless, the BG–PC derivative showed considerable promise, as sugarcane bagasse conversion close to 100% could be achieved within 96 h when the reaction medium was supplemented with

BG–PC. In terms of recyclability, conversion remained very stable up to the third cycle and decreased by less than 50% after five cycles.

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