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Covalent Immobilization of β-Glucosidase on Magnetic Particles for Lignocellulose Hydrolysis

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Abstract β-Glucosidase hydrolyzes cellobiose to glucose and is an important enzyme in the consortium used for hydrolysis of cellulosic and lignocellulosic feedstocks. In the present work, β-glucosidase was covalently immobilized on non-porous magnetic particles to enable re-use of the enzyme. It was found that particles activated with cyanuric chloride and polyglutaraldehyde gave the highest bead-related immobilized enzyme activity when tested with *p*-nitrophenyl-β-D-glucopyranoside (104.7 and 82.2 U/g particles, respectively). Furthermore, the purified β-glucosidase preparation from Megazyme gave higher bead-related enzyme activities compared to Novozym 188 (79.0 and 9.8 U/g particles, respectively). A significant improvement in thermal stability was observed for immobilized enzyme compared to free enzyme; after 5 h (at 65 °C), 36 % of activity remained for the former, while there was no activity in the latter. The performance and recyclability of immobilized β-glucosidase on more complex substrate (pretreated spruce) was also studied. It was shown that adding immobilized β-glucosidase (16 U/g dry matter) to free cellulases (8 FPU/g dry matter) increased the hydrolysis yield of pretreated spruce from ca. 44 % to ca. 65 %. In addition, it was possible to re-use the immobilized β-glucosidase in the spruce and retain activity for at least four cycles. The immobilized enzyme thus shows promise for lignocellulose hydrolysis.

Keywords Lignocellulose hydrolysis · Immobilization · Enzymes · Magnetic particles · Pretreated spruce

Introduction

There is an increasing demand for replacing petroleum-based products with environmentally sustainable biobased chemicals. Biochemicals produced from lignocellulosic biomass is

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currently one of the most topical subjects; however, there is an increasing realization that the great availability and low cost of this raw material [1] will be one of the key drivers in the future biobased economy. An important step for the production of lignocellulosic-derived chemicals is the conversion of cellulose to glucose, which can be achieved enzymatically by the combined action of endoglucanases, exoglucanases, and β-glucosidases [2]. There are commercial preparations containing mixes of these enzymes where the most extensively studied originates from *Trichoderma reesei* fermentations [3, 4]. However, the amount of β-glucosidase produced by *T. reesei* is insufficient resulting in incomplete hydrolysis of cellulose due to product inhibition by cellobiose for endoglucanases and exoglucanases [5]. Many cellulase preparations are therefore supplemented with additional β-glucosidase to increase hydrolysis rate. This will increase the already high enzyme costs for the hydrolysis process. Enzyme immobilization on particles could reduce the enzyme cost by improving operational stability of the enzyme and allowing re-use [6, 7]. Recycling of the enzyme utilizing common separation unit operations such as centrifugation or filtration may, however, be difficult when treating crude particulate containing lignocellulosic feedstocks. One approach to overcome the difficulty in recycling would be to use enzymes immobilized on small magnetically susceptible particles [8, 9]. By applying an external magnetic field, the immobilized enzymes could thus be magnetically separated before being reused in a subsequent hydrolysis cycle. Magnetic particles have previously been shown to enable rapid and highly selective separation from crude liquors [10, 11].

Immobilization of β-glucosidase has been reported previously using different support materials and varying attachment methods such as adsorption and covalent reaction between the enzyme and the support [12–17]. Although adsorption is the simplest method for immobilization, covalent linkage provides a much more stable attachment, thus minimizing enzyme leakage from the support. In previous studies on immobilization of β-glucosidase, the crude enzyme preparation Novozym 188 (β-glucosidase from *Aspergillus niger*) has been frequently used [13, 18]. However, this preparation contains impurities such as other enzymes/proteins which could potentially be attached to the particles thus reducing the final bead specific β-glucosidase activity (U/g particles).

The aim of the present work was to covalently immobilize a purified β-glucosidase on magnetic particles and examine how different immobilization conditions, such as activation chemistries, immobilization time, and enzyme purity, affect the bead-related activity (U/g particles). Characterization including enzyme kinetics, temperature optimum, and thermal stability for free and immobilized enzyme were studied. A second objective was to investigate whether the immobilized β-glucosidase could work on more complex lignocellulosic substrate (bisulfite-pretreated spruce) and retain enzyme activity in repeated hydrolysis cycles.

Materials and Methods

Immobilization of β-Glucosidase on Different Functionalized Magnetic Particles

During immobilization of β-glucosidase, different commercial, micron-sized (Ø=1 μm) superparamagnetic particles were studied. They were non-porous silica-based ones which differed primarily in their activation chemistries. They consisted of cyanuric chloride-activated (M-Cyanuric), polyglutaraldehyde-activated (M-PGL), carboxyl-activated (M-Carboxyl) (all from Chemicell, Berlin, Germany), tosyl-activated (M-TShort), and long-arm tosyl-activated (M-TLong) magnetic particles from Bioclone (San Diego, CA). M-TLong consisted of a

hydrophilic linker (18 carbon) terminated with a tosyl group. The β-glucosidase was obtained from Megazyme (Bray, Ireland).

The particles were washed twice with 0.1 M phosphate buffer (pH 7.4) prior to immobilization. Enzyme immobilization was performed in Eppendorf tubes by mixing 1 mg of particles with the enzyme (6 U of β-glucosidase), for 2 h at room temperature, in 0.5 ml 0.1 M phosphate buffer (pH 7.4). The immobilization procedure was similar for all particles except for M-Carboxyl. The M-Carboxyl particles were activated by carbodiimide using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) prior to enzyme immobilization. This was conducted by mixing (for 10 min at room temperature) 1 mg of M-Carboxyl particles with 0.5 ml 0.1 M MES (2-(*N*-morpholino)ethanesulfonic acid) buffer (pH 5.0) containing 20 mg EDC. Subsequently, the particles were washed and enzyme immobilization was performed by mixing (for 2 h at room temperature) the carbodiimide-activated particles with the enzyme in 0.5 ml 0.1 M MES buffer (pH 5.0). In all cases, immobilization was stopped by magnetic capture of the particles and washed twice with phosphate or MES buffer. Unreacted functional groups were blocked (gentle mixing for 30 min at room temperature) using a blocking buffer of 0.1 M phosphate buffer (pH 7.4) containing 2 % bovine serum albumin (BSA) and 0.05 % NaN_3.

Additional experiments were conducted where immobilization time and pH was varied. For M-Cyanuric and M-PGL, the effect of smaller particle size (Ø=0.5 μm) on bead activity was also examined.

Effect of Amount of Added Enzyme During Immobilization and Enzyme Origin

The effect of added amount of enzyme prior to immobilization was studied for M-Cyanuric and M-PGL particles using β-glucosidase from Megazyme. The amount of enzyme added prior to immobilization varied from 0.2 to 10 U per milligram of support.

For all experiments, β-glucosidase from Megazyme (Bray, Ireland) was used. However, the potential of a cheaper preparation, Novozym 188 (obtained from Novozymes, Bagsværd, Denmark), was also examined. β-Glucosidase from Megazyme is a purified product [19] from the crude enzyme preparation Novozym 188 (β-glucosidase from *A. niger*). Equal amounts of enzyme units (6 U β-glucosidase/mg support) of either Megazyme β-glucosidase or Novozym 188 were used during coupling to the magnetic particles M-Cyanuric and M-PGL.

Enzyme Assay and Protein Determination of Free and Immobilized BG

The activity of free or immobilized β-glucosidase (U/g particles) was assayed using *p*-nitrophenyl-β-D-glucopyranoside (PNPG, Sigma) based on a previously described method for free β-glucosidase [20]. The assay mixture contained 0.9 ml 5 mM PNPG in 50 mM sodium acetate buffer (pH 4.8) and an appropriate amount of free or immobilized β-glucosidase in 100 μl sodium acetate buffer. After incubation at 50 °C for 4 min with gentle mixing, the immobilized enzyme was magnetically separated using a simple ~0.4-T bar magnet. Two milliliters of 1 M Na_2CO_3 was immediately added to the supernatant in order to terminate the reaction of any enzyme which might remain in solution. The liberated *p*-nitrophenol (PNP) was measured at 405 nm and a standard curve of PNP was used as a reference. One unit of β-glucosidase activity (U) releases 1 μmol PNP per minute under the assay conditions.

The amount of attached protein to the magnetic particles was determined by measuring protein content before and subsequent to immobilization in washing buffer solution. Protein content was estimated by the Bradford method [21] using bovine serum albumin as standard.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using Runblue SDS gel 4–20 % from Expedeon (Cambridgeshire, UK). Protein samples were prepared by adding 5 % v/v mercaptoethanol and 1/4 vol 4× LDS sample buffer accompanied by heating at 95 °C for 10 min. The gel was stained by Coomassie Brilliant Blue (CBB R-250), destained, and subsequently scanned using CanonScan D660U (Canon Inc., Tokyo, Japan).

Characterization of Temperature Optimum, Thermal Stability, and Enzyme Kinetics

From the trials described above, M-Cyanuric particles were chosen for further characterization studies. Temperature optimum for free and immobilized β-glucosidase was determined within the temperature range of 40–80 °C. Thermal stability was examined by incubating free and immobilized β-glucosidase at 65 °C from 0 to 5 h. Aliquots were taken from the samples at different time intervals and subsequently assayed according to the described PNPG assay. Michaelis–Menten kinetics of free and immobilized β-glucosidase were determined by monitoring the initial hydrolysis rate of PNPG at concentrations within the range of 0.1–10 mM. K_m and V_{max} for free and immobilized were determined by Lineweaver–Burk plot.

Lignocellulose Hydrolysis Using Free Cellulase in Combination with Immobilized β-Glucosidase

In order to study the effect of immobilized β-glucosidase on more complex substrate, compared to PNPG, bisulfite-pretreated spruce (pretreatment conditions—0.8 % sulfuric acid and 20 % bisulfite, temperature—140 °C, time—10 h) was utilized. The spruce was kindly provided by Paper and Fibre Institute (Trondheim, Norway) and the composition was as follows (wt/wt DM): cellulose, 59.3 %; arabinoxylan, 2.9 %; galactoglucomannan, 8.3 %; acid-insoluble lignin, 11.3 %; and acid-soluble lignin, 3 %. Immobilized β-glucosidase (using M-Cyanuric particles) was combined with free cellulases (Celluclast 1.5L obtained from Novozymes) during the hydrolysis trials. The possibility of recycling the magnetic particles and retaining activity after a hydrolysis cycle was also examined (in total four hydrolysis campaigns were conducted). The trials were performed in 2-ml Eppendorf tubes using 1.5 % (w/v) dry matter (DM) of pretreated spruce suspended in 50 mM acetate buffer (pH 4.8). The mixture contained 0.05 % (w/v) NaN_3 to prevent microbial growth. The amount of added Celluclast 1.5L was 8 FPU (filter paper units)/g DM pretreated spruce and the amount of added immobilized activity (using M-Cyanuric particles) of β-glucosidase was 16 U/g DM pretreated spruce. One trial was also employed where only Celluclast 1.5L was added (8 FPU/g DM pretreated spruce) using the same conditions described above. The hydrolysis was performed at 50 °C with gentle mixing using a rotator. After 24 h of incubation, the immobilized β-glucosidase was magnetically separated using a magnetic bar and the amount of released reducing sugar in the supernatant was determined by the DNS (3,5-dinitrosalicylic acid) method using glucose as standard [22]. After one hydrolysis cycle (24 h), the particles were washed three times with 0.1 M phosphate buffer containing 0.5 % BSA. Subsequently, they were used for a second hydrolysis cycle using the same conditions described above. This was performed in total of four campaigns.

The hydrolysis yield was determined based on the total amount of released reducing sugar by sulfuric acid hydrolysis (LAP established by NREL [23]). The sulfuric acid hydrolysis was performed (in triplicates) by incubating 100 mg of dried spruce in 1 ml of 72 % sulfuric acid for 1 h at 30 °C. The content was then diluted to 4 % sulfuric acid with

distilled water and incubated at 121 °C for 1 h. Subsequently, the amount of released reducing sugar was determined by the DNS method.

Results and Discussion

Immobilization of β-Glucosidase on Different Functionalized Magnetic Particles

The purpose of this study was to covalently immobilize a purified β-glucosidase on magnetic particles and examine how different immobilization conditions, such as activation chemistries, immobilization time, and enzyme purity, affect the bead-related activity (U/g particles). A second objective was to investigate whether the immobilized β-glucosidase could work on more complex lignocellulosic substrate (pretreated spruce) and retain enzyme activity subsequent to a hydrolysis cycle.

Five different commercial, micron-sized, superparamagnetic, non-porous silica-coated particles were studied. They differed primarily in their activation chemistries since not only are different chemistries more reactive than others but the method of attachment to the protein and presence of spacer arms can be expected to influence the resultant activity.

Table 1 displays bead-related immobilized β-glucosidase activity (U/g particles) after coupling to the different particles used. The results demonstrate that it is possible to immobilize active β-glucosidase on the magnetic particles. It can be observed that M-Cyanuric and M-PGL yield substantially higher activities (79.0 and 75.3 U/g particles, respectively) compared to M-Carboxyl, M-TShort, and M-TLong after 2 h of coupling. A high bead-related activity is important to reduce the concentration and cost of support material in a large-scale application. As an example, a suspension containing 2 % (w/v) cellulose where 20 U of β-glucosidase is added per gram of cellulose would give a magnetic particle concentration of 5 g/l (based on bead activity of 79.0 U/g particles).

Longer incubation time (24 h) and higher coupling pH (from pH 5 to 7.0 for M-Carboxyl and pH 7.4 to 9.5 for M-Cyanuric, M-PGL, M-TShort, and M-TLong) were used in order to boost activity. From Table 1, it can be observed that increasing incubation time resulted in

Table 1 Comparison of the bead-related immobilized enzyme activity after coupling Megazyme β-glucosidase to magnetic particles activated with different functional groups

Magnetic particle type	pH during coupling					
	7.4[a]	9.5[b]	7.4[a]	5.0[c]	7.0[c]	5.0[c]
	Coupling time (h)					
	2	2	24	2	2	24
	Bead-related immobilized enzyme activity[d] (U/g particles)					
M-Cyanuric	79.0	73.8	104.7			
M-PGL	75.3	72.6	82.2			
M-Carboxyl				12.6	12.0	14.6
M-TShort	12.4	13.2	20.8			
M-TLong	10.5	11.5	14.1			

[a] Binding buffer: 0.1 M phosphate buffer

[b] Binding buffer: 0.1 M sodium carbonate buffer

[c] Binding buffer: 0.1 M MES buffer

[d] Substrate used was PNPG

increased bead-related enzyme activity, in particular for M-TShort and M-Cyanuric particles. The enzyme-loading capacity using M-Cyanuric particles and an incubation time of 24 h was determined to 7.8 mg protein/g particles. In addition to higher activity, increased coupling time could promote multipoint attachment, between enzyme and support, which has been reported to increase enzyme stability [24, 25]. By increasing the pH of the coupling buffer, it was expected that the covalent reaction with the support could be increased because of enhanced nucleophilic character of the amine groups of β-glucosidase. However, Table 1 shows that for both M-TShort and M-TLong there was only a slight increase in enzyme activity while for M-Cyanuric, M-PGL, and M-Carboxyl, the activity decreased.

It was thought that M-TLong may display higher enzyme activities compared to M-TShort because of the long spacer arm, thus leading to less steric hindrance for the substrates approach to the active site. However, Table 1 indicates that M-TLong gave a lower activity compared to M-TShort. The effect of using a hydrophilic linker attached to the particle may, however, be more pronounced when using substrates with higher molar mass or which are insoluble, as would be the case for immobilized endoglucanases and exoglucanases. The PNPG substrate is only 0.3 kDa in size and would be able to easily diffuse to the active site [15]. The effect of decreasing the particle size was studied for M-Cyanuric and M-PGL particles since smaller particles could promote higher surface area per gram of particles. Using a particle size of 0.5 μm instead of 1 μm increased bead-related activity by 16 % for M-Cyanuric particles. For M-PGL, no significant increment was observed.

Effect of Amount of Added Enzyme During Immobilization and Enzyme Origin

For M-Cyanuric and M-PGL particles, the effects of varying the amount of enzyme units added during immobilization was studied. In terms of process, economizing the amount of added enzyme is an important factor to consider, and it can be observed in Fig. 1 that the bead-related enzyme activity for M-Cyanuric was higher than M-PGL particles at all enzyme loadings. It was also observed that for both particle types, there was only a slight improvement when more than 6 U of free enzyme/mg particles was added during immobilization, suggesting that the coupling sites on the particles were saturated with enzyme.

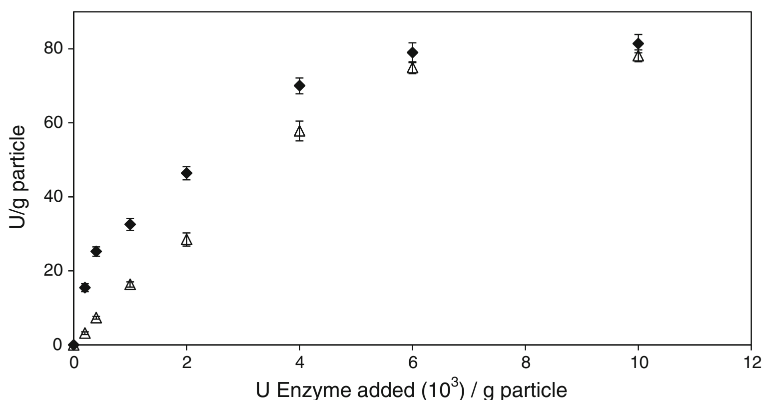

Fig. 1 Bead related activity (U/g particles) after coupling different amounts of Megazyme β-glucosidase to M-Cyanuric (*filled diamonds*) and M-PGL (*open triangles*) particles. Data and *error bars* represent average and standard deviation, respectively, of three replicate experiments

A cheaper alternative to Megazyme β-glucosidase, i.e., Novozym 188, was also examined for immobilization. Equal amounts of enzyme units (6 U β-glucosidase/mg particles) were used during coupling to M-Cyanuric and M-PGL particles, and the results are shown in Fig. 2. It can be observed that there is almost a 10-fold lower enzyme activity when using Novozym 188 compared to Megazyme β-glucosidase for both M-Cyanuric and M-PGL particles. This difference correlates well to the difference in specific activity of free Megazyme β-glucosidase and Novozym 188 which was determined to be 54.6 and 8.3 U/mg protein, respectively, using the PNPG assay. The values of bead-related activity using Novozym 188 (9.8 and 8.5 U/g particles for M-Cyanuric and M-PGL particles, respectively) are slightly higher compared to a previous study by Tu et al. [16]. They covalently immobilized Novozym 188 on Eupergit C (a non-porous epoxy-activated support) and obtained a bead-related immobilized β-glucosidase activity of 3.5 U/g particles. Novozym 188 is a crude enzyme preparation while Megazyme β-glucosidase is a purified preparation [19, 26]. The primary amine groups exposed on the surface of other enzymes besides β-glucosidase are most likely also covalently linked to the particles during the immobilization step, thus reducing the final bead-specific β-glucosidase activity of the Novozym 188. In addition, the difference in specific activity between Megazyme β-glucosidase and Novozym 188 is in fact even higher after enzyme immobilization, which could be due to higher affinity of the impurities in Novozym 188 to the particles, compared to β-glucosidase. The difference in enzyme purity is displayed by SDS–PAGE (inset in Fig. 2) of free Megazyme β-glucosidase and Novozym 188. For Megazyme β-glucosidase (lane 1), it can be seen that two clear bands are visible; ca. 120 kDa and 70 kDa representing β-glucosidase and BSA, respectively (BSA is added to promote stability during storage). For Novozym 188 (lane 3), it can be observed that β-glucosidase is present and there are three additional bands with molecular weights of about 60, 80, and 105 kDa. When overloading Novozym 188 (lane 4), it can be observed that three protein bands appear within the molecular weight range of 25–35 kDa.

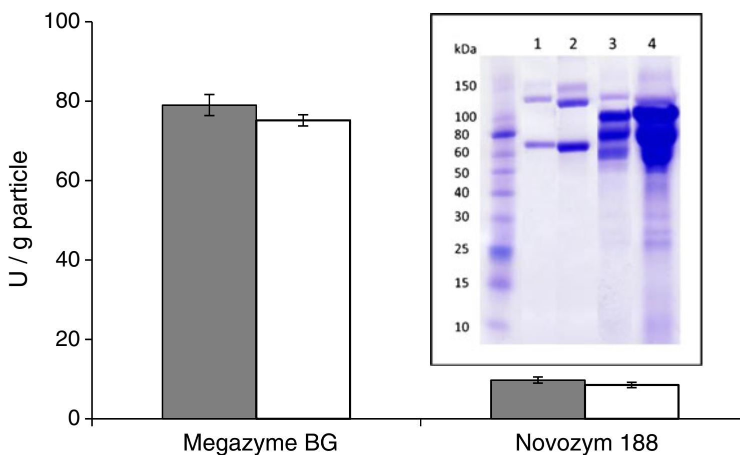

Fig. 2 Bead-related immobilized enzyme activity (U/g particles) when Megazyme β-glucosidase or Novozym 188 have been covalently attached to M-Cyanuric (*filled squares*) or M-PGL (*open squares*) particles. During the immobilization procedure, equal amounts of enzyme units were added (6 U/mg support). Data and *error bars* represent average and standard deviation, respectively, of three replicate experiments. The inset shows SDS–PAGE of Megazyme β-glucosidase (*lane 1*), overloaded Megazyme β-glucosidase (*lane 2*), Novozym 188 (*lane 3*), and overloaded Novozym 188 (*lane 4*)

Characterization of Temperature Optimum, Thermal Stability, and Enzyme Kinetics

Based on the preceding data, M-Cyanuric particles were chosen for further characterization studies. The temperature optimum for free and immobilized β-glucosidase was determined within the temperature range of 40–80 °C. Figure 3 shows the relative activity as a function of temperature for free and immobilized β-glucosidase on M-Cyanuric particles. It can be observed that there is a slight increase in temperature optimum for immobilized β-glucosidase (65 and 70 °C for free and immobilized β-glucosidase, respectively). This shift in temperature optimum could possibly be explained by an increased thermal stability resulting from immobilization of the β-glucosidase. Two competing factors exist when performing a temperature optimum study: increased catalytic activity with increasing temperature and increased enzyme denaturation with increasing temperature. After the optimum temperature is reached, the denaturing effect of temperature is greater than its effect on reaction rate [27].

In order to study thermal stability, or resistance to enzyme denaturation, free and immobilized β-glucosidase was incubated at 65 °C from 0 to 5 h, and the results are shown in Fig. 4. It can be observed that after 1 h of incubation, the activity for free and immobilized β-glucosidase has been decreased to 40 % and 74 % of its initial enzyme activity, respectively. After 5 h of incubation, the residual activity for free enzyme is close to zero, while there is still activity for immobilized β-glucosidase (about 36 % residual activity). The figure demonstrates that there is significant improvement in thermal stability due to immobilization of the enzyme. This result is in agreement with previous work by Calsavara et al. [13] where Novozym 188 was covalently immobilized on porous silica particles. They observed that the thermal stability was 18.8 times higher for immobilized β-glucosidase compared to free β-glucosidase. The increased thermal stability could possibly be explained by multipoint covalent attachment between β-glucosidase and the support which increases the conformational stability and rigidity of the enzyme molecule [25, 28].

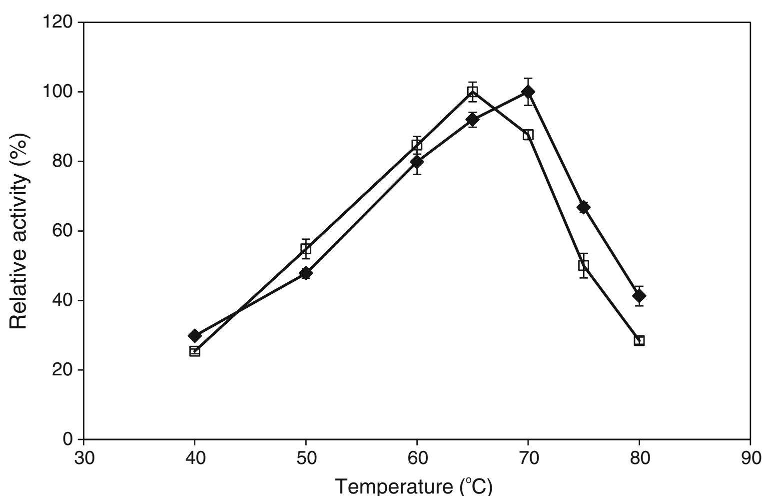

Fig. 3 Activity for free (*open squares*) and immobilized (*filled diamonds*) β-glucosidase as a function of temperature (within the temperature range of 40–80 °C). The maximum activity was normalized to 100 % and in the case of the free and immobilized enzyme were 97.5 U/mg protein and 219.1 U/g particles, respectively. Data and *error bars* represent average and standard deviation, respectively, of three replicate experiments

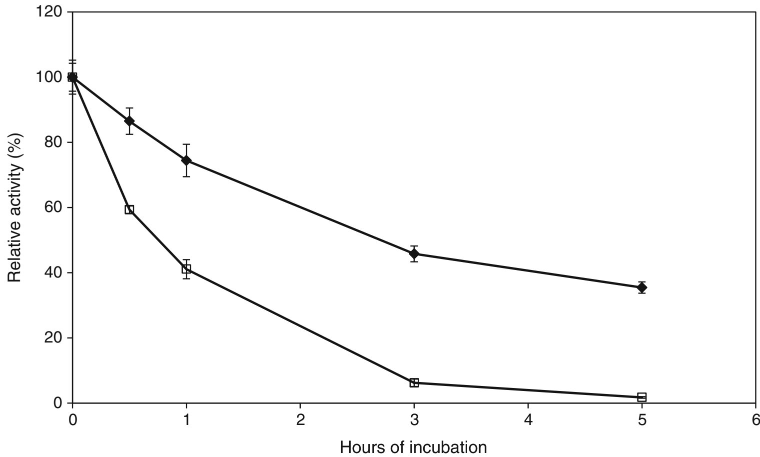

Fig. 4 Relative activity as a function of incubation time (h) for free (*open squares*) and immobilized (*filled diamonds*) β-glucosidase. Incubation temperature was 65 °C. Data and *error bars* represent average and standard deviation, respectively, of three replicate experiments

Michaelis–Menten kinetics for free and immobilized β-glucosidase was determined by monitoring the initial hydrolysis rate of PNPG at concentrations within the range of 0.1–10 mM (Fig. 5). During the standard PNPG assay described in "Materials and Methods", 5 mM PNPG was used. It can be observed in Fig. 5 that this substrate concentration is sufficiently high for both free and immobilized β-glucosidase to reach $V_{\max}$. A slight reduced hydrolysis rate was observed when increasing PNPG concentration from 5 to 10 mM. This trend has been reported previously for β-glucosidase from *A. niger* and was explained by substrate inhibition or transglycosylation [29, 30]. The Michaelis–Menten constants for free and immobilized enzyme were determined by Lineweaver–Burk plot

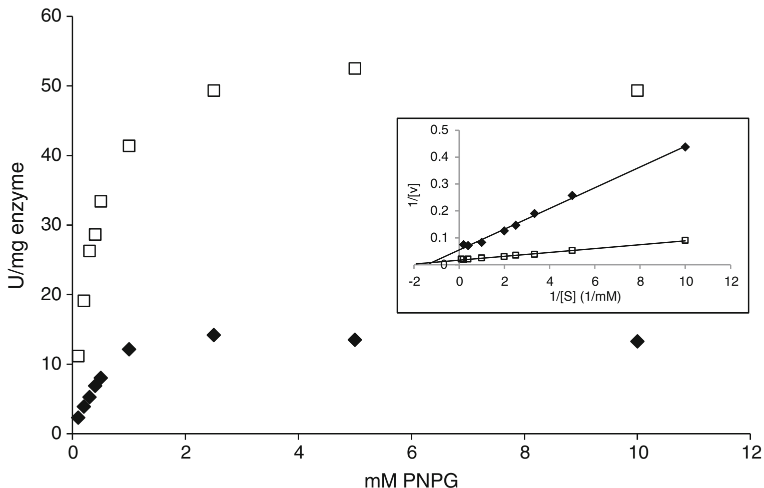

Fig. 5 Michaelis–Menten plot for free (*open squares*) and immobilized (*filled diamonds*) β-glucosidase. The inset shows the Lineweaver–Burk plot of initial hydrolysis rate versus fixed substrate concentration (0.1–10 mM PNPG). Data points are averages of duplicate measurements

(inset in Fig. 5). V_{max} and K_m values for free β-glucosidase were determined to 58.5 U/mg protein and 0.41 mM, respectively, while for immobilized β-glucosidase these values were 18.1 U/mg protein and 0.71 mM. The differences in apparent K_m and V_{max} between free and immobilized β-glucosidase could be attributed to alteration of the enzyme structure upon immobilization and/or due to lower accessibility of the substrate to the active site for the immobilized enzyme [16, 31, 32].

Lignocellulose Hydrolysis Using Free Cellulase in Combination with Immobilized β-Glucosidase

PNPG is a synthetic substrate and the assay mixture does not contain any insolubles during hydrolysis. In order to study the effect of the immobilized β-glucosidase on more complex substrate, pretreated spruce was used (composition reported in "Materials and Methods"). Immobilized β-glucosidase (using M-Cyanuric particles) was combined with free cellulases (Celluclast 1.5L obtained from Novozymes) during the hydrolysis trials. Figure 6 shows that the hydrolysis yield using only Celluclast 1.5L and Celluclast 1.5L with added immobilized β-glucosidase is 44 % and 65 %, respectively. Thus, the results confirm that the immobilized β-glucosidase can be used on more complex lignocellulosic substrate such as pretreated spruce. After one hydrolysis cycle, the immobilized β-glucosidase was magnetically separated, washed, and then used for a new hydrolysis cycle with fresh substrate and cellulase. Figure 6 shows that the immobilized β-glucosidase could be used, to increase the hydrolysis rate of free cellulases, for at least four hydrolysis cycles. However, it can be observed that after the fourth cycle, the effect on hydrolysis yield of added immobilized β-glucosidase has decreased by 52 % from the first hydrolysis cycle. The loss in activity could be due to deactivation of immobilized β-glucosidase during each hydrolysis cycle or to loss of magnetically immobilized enzyme particles during the magnetic separation and re-

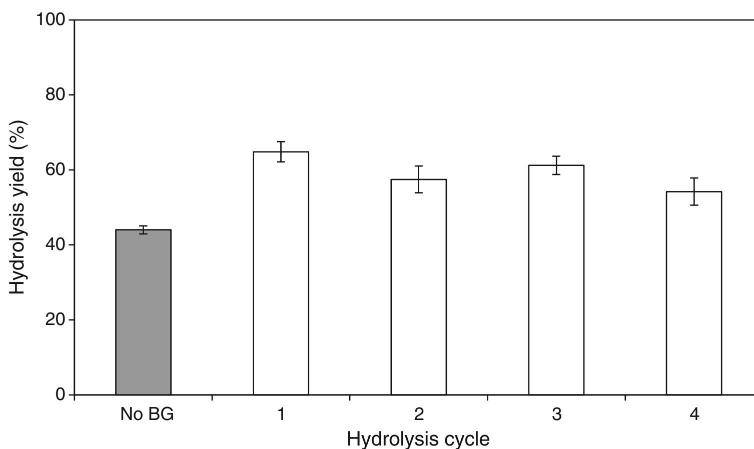

Fig. 6 Hydrolysis yield of pretreated spruce using Celluclast 1.5L in combination with immobilized β-glucosidase (immobilized on M-Cyanuric particles). Hydrolysis yield was determined as liberated reducing sugars divided by the total amount of reducing sugar (total amount of reducing sugar was determined to 63 % wt/wt DM). No β-glucosidase (BG)=only Celluclast 1.5L. One hydrolysis cycle was performed for 24 h using 1.5 % (w/v) DM spruce, pH 4.8 (50 mM acetate buffer) at 50 °C. Amount of added Celluclast 1.5L and immobilized β-glucosidase was 8 FPU/g DM and 16 U/g DM, respectively. Fresh Celluclast 1.5L was added to each new hydrolysis cycle

dispersion steps [17, 33]. Preliminary data suggest the latter; by measuring the remaining iron content (Fe^{2+} and Fe^{3+}) spectrophotometrically, it was observed that the major contribution to decreased enzyme activity was due to loss of magnetic particles.

Conclusion

Magnetic particles activated with cyanuric chloride and polyglutaraldehyde are promising for immobilization of β-glucosidase (yielding bead-related immobilized enzyme activity of 104.7 and 82.2 U/g particles, respectively). Immobilization leads to a significant increase in thermal stability of the enzyme at 65 °C. Adding immobilized β-glucosidase to free cellulases increases the hydrolysis rate of pretreated spruce. Furthermore, it is possible to recycle the immobilized β-glucosidase and retain activity for at least four hydrolysis campaigns. The immobilized enzyme thus shows promise for lignocellulose hydrolysis.

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