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Immobilization of cellulases on magnetic particles to enable enzyme recycling during hydrolysis of lignocellulose

Ph.D. thesis

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Summary

There is an urgent need to replace petroleum-based fuels and chemicals with more environmentally sustainable options since oil contributes to a net production of greenhouse gases and is a limited resource. Lignocellulosic biomass is currently one of the most extensively studied feedstocks for biochemicals and biofuels production because of the great abundance of the feedstock and the fact that it is a waste material and does not directly compete with food production. Lignocellulose consists of cellulose (the most prevalent component), hemicellulose and lignin and the polymeric sugars (in cellulose and hemicellulose) can be enzymatically hydrolyzed into monomers and subsequently fermented to bioethanol or another desirable biochemical. One of the main costs and obstacles in making the bioprocess economically viable is the costs of cellulases which catalyze the hydrolysis of cellulose into glucose. One approach to decrease the costs of the cellulases could be to immobilize the enzymes on particles and thereby enable enzyme re-use. However, recycling of immobilized cellulases using common separation unit operations such as centrifugation or filtration may be difficult when dealing with lignocellulosic feedstocks containing insolubles. This could potentially be overcome by immobilizing the cellulases on magnetically susceptible particles. Consequently, the immobilized cellulases could be magnetically recovered and recycled for a new cycle of enzymatic hydrolysis of cellulose.

The main objective of this thesis was to examine the possibility of immobilizing cellulases on magnetic particles in order to enable enzyme re-use. Studies at lab and pilot scale (20 L) were conducted using model and real substrates. In paper I and III beta-glucosidase or a whole cellulase mixture was covalently immobilized on commercial, but expensive, magnetic particles activated with different chemistries. It was observed that the highest immobilized enzyme activities were obtained using magnetic particles activated with cyanuric chloride. In paper II biotinylated recombinant beta-glucosidase was produced and immobilized on commercial magnetic particles coated with streptavidin. The procedure enabled simultaneous purification and immobilization from crude cell lysate because of the very strong interaction and high affinity between biotin and streptavidin. A third method of immobilizing enzymes was employed in paper IV where two types of magnetic anion exchange particles were used to immobilize beta-glucosidase through electrostatic interactions. For both covalent coupling and adsorption (anion exchange binding)

between enzyme and support the specific activity (U/mg protein) of immobilized enzyme was lower compared to the free form, while for enzyme immobilization using the biotin-streptavidin system the specific activity increased by 6.5-fold upon immobilization compared to the crude cell lysate.

Following enzyme immobilization the possibility of recycling the enzyme was examined using both synthetic substrates (soluble) and real lignocellulosic biomass (containing insolubles such as lignin, hemicellulose and non-hydrolyzed cellulose). The most promising particles for recycling were the anion exchange magnetic particles from Orica Watercare (MIEX® particles) since they promoted rapid magnetic separation and very low interaction with residual insolubles. In addition to these features, they are extremely cheap. It was also possible to strip adsorbed enzyme under special conditions and re-charge the particles with new fresh enzyme (which could decrease the cost associated with purchase of base particles). For these reasons, 400 g of the magnetic MIEX® particles were used for enzyme immobilization and enzyme recycling during the 20 L pilot scale study conducted in paper V. A new type of high gradient magnetic separator, a magnetically enhanced centrifuge, was employed and it was possible to recover the immobilized enzyme and separate the magnetic particles from residual cellulose in pilot scale, before using them in 3 subsequent 20 L hydrolysis cycles. The results in this thesis thus demonstrate that cheap magnetic immobilized cellulases can be used for repeated hydrolysis cycles at pilot scale and demonstrate the potential for use in large scale applications such as in the production of lignocellulosic derived biochemicals.

The thesis is based on the following papers (referred by their roman numerals in the text):

I.

Alftrén, J., Hobley, T.J. (2013) Covalent immobilization of β-glucosidase on magnetic particles for lignocellulose hydrolysis. *Applied Biochemistry and Biotechnology,* 169, 2076-2087.

II.

Alftrén, J., Ottow, K., Hobley, T.J. (2013) *In vivo* biotinylation of recombinant beta-glucosidase enables simultaneous purification and immobilization on streptavidin coated magnetic particles. *Journal of Molecular Catalysis B: Enzymatic,* 94, 29-35.

III.

Alftrén, J., Hobley, T.J. (2013) Immobilization of cellulase mixtures on magnetic particles for hydrolysis of lignocellulose and ease of recycling. *Submitted to Biomass & Bioenergy*.

IV.

Alftrén, J., Søndergaard, W., Hobley, T.J. (2013) Immobilization of beta-glucosidase by adsorption on anion exchange magnetic particles enables enzyme re-use during hydrolysis of pretreated wheat straw. *In preparation.*

V.

Alftrén, J., Søndergaard, W., Hobley, T.J. (2013) Pilot scale demonstration of re-use and separation of magnetically immobilized beta-glucosidase. *In preparation.*

My contribution to the papers:

Paper I-III: I performed all the experimental work, took active part in data evaluation and wrote the major part of the paper.

Paper IV: I performed part of the experimental work, took active part in data evaluation and wrote the major part of the paper.

Paper V: I performed part of the experimental work, took active part in data evaluation and wrote part of the paper.

Oral and poster presentations during the thesis

Oral presentations

Alftrén, J., Hobley, T.J. Immobilization of cellulase mixtures on magnetic particles for hydrolysis of lignocellulose and ease of recycling. *21st European Biomass Conference and Exhibtion,* Copenhagen, Denmark, 3rd - 7th of June 2013.

Alftrén, J., Ottow, K.E., Hobley, T.J. A simple approach for generating reusable enzymes: *In vivo* biotinylation of recombinant beta-glucosidase, simultaneous purification and immobilization on streptavidin coated magnetic particles. *21st European Biomass Conference and Exhibtion,* Copenhagen, Denmark, 3rd - 7th of June 2013.

Alftrén, J., Hobley, T.J. Re-use of magnetically immobilized β-glucosidase for hydrolysis of pretreated spruce. *Technoport RERC (Renewable Energy Research Conference) ,* Trondheim, Norway, 16 – 18th of April 2012.

Alftrén, J., Hobley, T.J. Immobilization of β-glucosidase on magnetic particles for lignocellulose hydrolysis. *International Symposium on Alcohol Fuels, ISAF XIX,* Verona, Italy, 10 – 14th of October 2011.

Poster presentations

Alftrén, J., Mansha, I., Hobley, T.J. Cellulase immobilization on magnetic particles enables enzyme recycling during lignocellulose hydrolysis. *34th Symposium on Biotechnology for Fuels and Chemicals,* New Orleans, USA, 30th of April – 3rd of May 2013.

Alftrén, J., Hobley, T.J. Immobilization of cellulases on magnetic particles enables enzyme re-use during hydrolysis of pretreated spruce. *4th Nordic Wood Biorefinery Conference*, Helsinki, Finland, 23rd – 25th of October 2012.

Papers not included in the thesis

Runyon, J.R., Nilsson, L., Alftrén, J., Bergenståhl, B. (2013) Characterization of oat proteins and aggregates using asymmetric flow field-flow fractionation. *Analytical and Bioanalytical Chemistry*, 405, 6649-6655.

Alftrén, J., Peñarrieta, J.M., Bergenståhl, B., Nilsson, L. (2012) Comparison of molecular and emulsifying properties of gum arabic and mesquite gum using asymmetrical flow field-flow fractionation. *Food Hydrocolloids,* 26, 54-62.

Gosling, A., Alftrén, J., Stevens, G.W., Barber, A.R., Kentish, S.E., Gras, S. (2009) Facile pretreatment of *Bacillus circulans* β-Galactosidase increases the yield of galactosyl oligosaccharides in milk and lactose reaction systems. *Journal of Agricultural and Food Chemistry*, 57, 11570–11574.

Contents

Appendix 1: Recombinant production of a biotin-6His-tag beta-glucosidase in *E. coli* – Optimization of protein expression and purification

1. Background

It is critical to replace petroleum-based fuels and chemicals with more sustainable alternatives since oil is a limited resource and contributes to a net production of greenhouse gases. Furthermore, the oil supply is uncertain and often linked with political tensions. In the biorefinery concept sugars from renewable biomass, of different origin, are utilized and converted to biofuels and biochemicals such as bioethanol, 1,4-succinic acid, 3-hydroxy propionic acid and aspartic acid [1]. For example, glucose could be fermented, by *E. coli*, to 1,4-succinic acid and this platform biochemical can be used as a building block for the production of nylon-like polymers [2, 3]. As transportation fuel, bioethanol is considered as a good alternative and could be blended with gasoline (up to 30% of ethanol) or be utilized as a sole fuel for vehicles with modified engines [4]. Bioethanol could be produced from biomass resources comprising starch and sugar crops or lignocellulosic material. Currently, the main production of bioethanol is based on the two former biomass feedstocks (referred to as first generation bioethanol), however, the method is highly criticized and debated since it competes for land and water use during food production, and is thus linked with the concern of yielding increased food prices [5, 6]. Consequently, this process is not an optimal alternative in terms of sustainability and this has altered the focus to other more viable options. Lignocellulosic material is a promising sustainable feedstock since it does not directly compete with food crops and represents one of the most abundant resource of renewable biomass on Earth [7]. It can be obtained from forestry (such as saw mills and pulp and paper industry) and agricultural waste (such as corn stover, sugarcane bagasse and wheat straw) and is therefore regarded as a low-cost substrate. Because of these beneficial attributes, lignocellulosic biomass is currently one of the most extensively studied feedstock for producing biofuels and biochemicals. The development of bioethanol production from lignocellulose (referred to as second generation bioethanol) has at present reached the point where the first large-scale facilities are being installed and completed for commercial production. However, it is crucial to further develop the technology in order to decrease, in comparison to gasoline, the high production costs.

1.1 Lignocellulose

One of the main obstacles during production of second generation bioethanol, compared to first generation where the polymeric sugars are readily available for hydrolysis, is the recalcitrant and insoluble lignocellulosic biomass. Lignocellulose is found in the plant cell wall and it promotes

structure and acts as an environmental barrier to the plant cell. The major components in lignocellulose are cellulose, hemicellulose and lignin representing about (composition is dependent on plant species) 35-50%, 20-35% and 5-30% of the biomass, respectively [8, 9]. Cellulose is a linear polymer of glucose linked by beta-1,4-glycosidic bonds and is the main constituent in lignocellulose. The degree of polymerization of cellulose chains varies between 100 and up to 14,000 glucose units [10], and the polymers are oriented in parallel forming strong intermolecular interactions through hydrogen bonds, van der Waal´s forces and hydrophobic interactions [11]. Through the intermolecular forces crystalline cellulose fibres (containing parts of amorphous regions) are formed from several assembled polymers and this make cellulose water insoluble and recalcitrant to hydrolysis. In contrast to cellulose, hemicellulose is a much more complex and heterogeneous polysaccharide consisting of pentoses, hexoses and sugar acids [12]. The polymer is commonly branched and it does not possess any crystalline structure and is thus more easily converted into its monomeric sugars. The functional role in plants is to strengthen the cell wall by interacting with cellulose and lignin. The main hexoses consist of glucose, mannose and arabinose, while the pentoses mainly consist of xylose and arabinose. The structure and composition of the monomeric sugars vary widely between different plant species. For example, in soft wood (e.g. spruce) the main sugar is mannose and for grasses (e.g. wheat straw) the main sugar is xylose. The third component in lignocellulose is lignin and it is a complex hydrophobic polymer of aromatic alcohols. In the plant cell wall, lignin forms covalent bonds with hemicellulose resulting in a cross-linked network surrounding the crystalline cellulose and thereby decreasing the potential of hydrolyzing cellulose even more.

1.2 Production of lignocellulosic bioethanol

Production of second generation bioethanol commonly involves the step of pretreatment, enzymatic hydrolysis, fermentation and distillation (Figure 1). Pretreatment is performed in order to modify the structure of the lignocellulosic material rendering cellulose more susceptible to enzymatic hydrolysis. A good pretreatment method should be cost-effective, generate a digestible substrate and form low amount of inhibitors to the fermentation step. Generally, pretreatment involves a physical step, using mechanical procedures such as milling or extrusion, in order to reduce the size of the material (and thereby increase the surface area of the material) and decrease the crystallinity of cellulose [13]. This is followed by a chemical, physico-chemical or biological pretreatment step.

Chemical treatment could be performed using for example sulfuric acid (acid pretreatment) or sodium hydroxide (alkaline pretreatment) where the former removes (hydrolyzes) the hemicellolusic fraction while the latter remove part of the lignin fraction. In a novel method of chemical pretreatment, ionic liquids are employed which make cellulose amorphous and porous resulting in substantial improvement in the subsequent enzymatic hydrolysis step. However, the main drawback of this method, so far, is the high costs of the ionic liquids [14, 15]. Physico-chemical treatment combines physical and chemical processes to alter the lignocellulosic structure and the most common procedures are steam explosion, ammonia fiber explosion and liquid hot water [16]. Biological pretreatment is the third group and employs lignin-degrading migroorganisms. Despite the advantages of no chemical addition and mild conditions little attention has been paid to this method mainly because of the slow process and the risk of microorganism consuming the sugars in cellulose and hemicellulose [17, 18].

The next step during production of lignocellulosic bioethanol is the enzymatic hydrolysis of cellulose and hemicellulose into C6 and C5 soluble sugars using enzymes. Depending on the chosen pretreatment method, the hemicellulose fraction is often already hydrolyzed during the pretreatment step (such as for acid treatment) and in those cases enzymes for hemicellulose hydrolysis are not needed. The enzymatic hydrolysis of cellulose is employed by cellulases consisting of endoglucanases, exoglucanases (or cellobiohydrolases) and beta-glucosidase [19, 20]. Enzymatic hydrolysis of hemicellulose is conducted using hemicellulases and since hemicellulose is a heterogeneous polysaccharide many different enzymes could be utilized. In addition to hydrolyzing hemicellulose into monomeric sugars, hemicellulases increase the hydrolysis rate of cellulose by rendering cellulose more accessible to cellulases. The most frequently studied hemicellulases are xylanases hydrolyzing xylan (polymeric backbone in hemicellulose) into xylose [21]. Examples of other hemicellulases that could be used for hydrolyzing hemicellulose are mannanases, galactosidases and arabinanases [13, 22]. Since cellulose is comprised of glucose and is the most prevalent component in lignocellulose the main research focus of the enzymatic hydrolysis step is to develop efficient cellulases. There are numerous parameters which affect enzymatic hydrolysis of lignocellulose and some of the most important are pH, temperature, enzyme loading, specific activity of the enzyme, crystallinity and accessibility of cellulose and lignin content [23]. These matters will be addressed in more detail later on in the thesis. Hydrolysis of lignocellulose into monomeric sugars could also be performed using dilute acid hydrolysis [24]. However, this process features drawbacks such as harsh conditions, low yield of cellulose to glucose conversion and

formation of inhibitory compounds (to the subsequent fermentation step). Consequently, the most promising hydrolysis method is the enzymatic approach utilizing cellulases and hemicellulases.

After hydrolysis the sugars are fermented to ethanol using a suitable microorganism, commonly the yeast *Saccharomyces cerevisiae* is used. It is important to recognize that all process steps prior to fermentation will affect the fermentation performance and especially the pretreatment step could generate many compounds (such as furfural and hydroxymethyl furfural) which are inhibitory to the microorganism. The optimal microorganism during fermentation should possess tolerance to high ethanol concentration and inhibitors formed during pretreatment, ability to ferment all sugars (both C6 and C5 sugars) and result in high ethanol yields and production rates [25]. For *S. cerevisae* the preferred substrate is glucose and the wild type strain is not able to ferment xylose (the second most abundant sugar in lignocellulose) to ethanol. In order to have a cost-effective bioprocess it is important to convert as much of the sugars as possible to ethanol and this has led to the development of recombinant strains of *S. cerevisiae* able to co-ferment glucose and xylose to ethanol [26, 27]. In Figure 1, the conventional method of performing enzymatic hydrolysis and fermentation is depicted, namely SHF: Separate Hydrolysis and Fermentation. An alternative process setup to SHF is SSF (simultaneous saccharification and fermentation) where the two steps are combined and carried out in the same vessel. The main advantage of SHF is the possibility of performing the enzymatic hydrolysis (commonly a temperature of 50ºC) and fermentation (commonly a temperature of 30-35ºC) at their optimal conditions. However, during enzymatic hydrolysis the enzymes are inhibited by the end products which decrease the rate of hydrolysis. This problem is overcome in the SSF mode since the sugars produced are continuously removed because of fermentation of these by the microorganism. Another advantage of SSF compared to SHF is the reduced capital costs since only one reactor tank is needed.

The last step is purification where ethanol is separated from water and this is normally carried out by distillation. This is an energy-demanding step and it is therefore important that the ethanol titer after the fermentation step is high (preferably above 4%) in order to obtain a cost-effective distillation step [28]. The by-product lignin does not contain any fermentable sugars and could instead be burned and used for heat and power to the bioethanol/biorefinery plant or as a source for producing high-value products [8].

It should be emphasized that the described method is not limited to bioethanol production; the principle is very similar for producing other lignocellulosic derived biochemicals comprising the

steps of saccharification of the polymers into sugars (by pretreatment and enzymatic hydrolysis) followed by microbial fermentation of sugars to the desired biochemical and then performing a suitable downstream processing step.

Substrate → Pretreatment → Enzymatic hydrolysis → Fermentation → Distillation → Bioethanol / Lignin
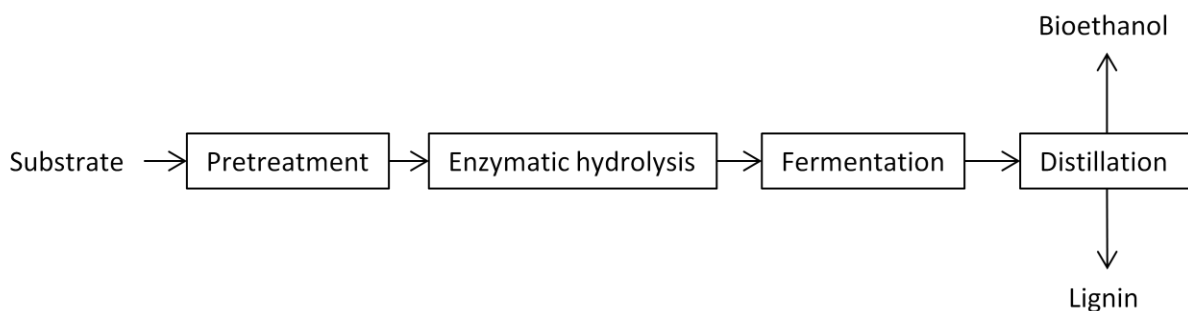
**Figure 1.** Process scheme for production of lignocellulosic bioethanol.

1.3 Objectives of the thesis

The main contributors to the production cost of lignocellulosic bioethanol and biochemicals are the costs of the feedstock, pretreatment and enzymes [5, 29, 30]. The technology needs to be further improved in order to be cost competitive with petroleum-based products. Thus, the main objective of my thesis was to investigate the possibility of immobilizing cellulases on magnetic particles in order to enable re-use and thereby decrease the costs of the enzymes. Different approaches of immobilizing the enzyme were studied and the biochemical properties of free and immobilized enzyme were examined. The performance of the immobilized enzyme was tested on both synthetic substrates and on real pretreated lignocellulosic materials. The major part of the study focused on immobilization of beta-glucosidase (one of the three cellulases) but the work also included studies on immobilization of whole cellulase mixtures. In all papers included the ability to recycle the immobilized enzyme was studied since this is the main benefit gained from immobilizing an enzyme. The major part of the work was carried out in lab-scale experiments and the most promising approach was scaled up and tested in pilot scale.

2. Cellulases

Cellulases catalyze the hydrolysis of the insoluble and recalcitrant cellulose into soluble glucose. As already described they consist of endoglucanases (EC 3.2.1.4), exoglucanases or cellobiohydrolases (EC 3.2.1.91) and beta-glucosidases (3.2.1.21) [19, 20] and their action on cellulose are depicted in Figure 2. Endoglucanases randomly hydrolyze internal beta-1,4 glycosidic bonds of regions of low crystallinity of cellulose. Exoglucanases cut cellobiose units from the ends of cellulose chains and beta-glucosidases hydrolyze cellobiose into two molecules of glucose [31, 32]. It should be mentioned that beta-glucosidase is not strictly a cellulase since the preferred substrate is cellobiose and not cellulose. However, it is normally included in the term 'cellulases' because it is needed to obtain the complete conversion of cellulose into the monomeric glucose. The structure of endo- and exoglucanases commonly consist of two domains: a catalytic core and a cellulose binding domain (CBD) connected by a flexible linker. The CBD binds to cellulose in order to bring the catalytic site close to the cellulose chain and thereby facilitating the hydrolysis of cellulose [33, 34]. All three cellulases act synergistically and it is important that the ratio between the three enzyme types is optimized in order to obtain good conversion rates. For example, low amounts of beta-glucosidase will result in cellobiose accumulation and in turn decrease hydrolysis rate since endoglucanase and exoglucanase are inhibited by cellobiose. Beta-glucosidase is inhibited by glucose and is thereby affected by the substrate concentration. In addition to find an optimal ratio the individual enzymes should preferably have high specific activity (Unit/mg enzyme), high thermostability and low susceptibility of being inhibited by cellobiose and glucose. Some desirable attributes of the substrate (cellulose/lignocellulose) is high accessibility to cellulose, low lignin content, low crystallinity and high porosity of the cellulose. Production of cellulases occurs mostly in fungi and bacteria and the most extensively studied organism originates from the fungi *Trichoderma reesei* [35]. However, the amount of beta-glucosidase produced by *T. reesei* is insufficient resulting in incomplete hydrolysis of cellulose due to cellobiose inhibition. Cellulase preparations from *T. reesei* are therefore often boosted with additional beta-glucosidase to increase the hydrolysis rate.

The activity of the cellulases can be quantified by different methods and the most common method to determine the overall cellulase activity is the filter paper assay where a filter paper strip (consisting of cellulose) is used as substrate and the amount of released reducing sugars is detected [36]. In paper III both overall cellulase activity and individual activities were determined of a cellulase mixture. For the individual enzyme activities the substrates azo-carboxymethyl cellulose,

p-nitrophenyl-beta-D-cellobioside and *p*-nitrophenyl-beta-D-glucopyranoside were used for measuring endoglucanase, exoglucanase and beta-glucosidase activity, respectively [37, 38].
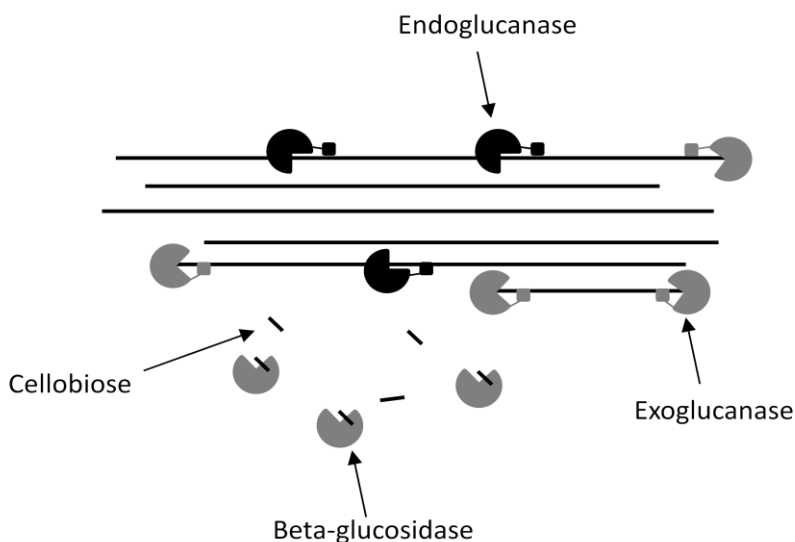
Figure 2. Schematic illustration of the action of cellulases (endoglucanase, exoglucanase and beta-glucosidase) for hydrolysis of cellulose.

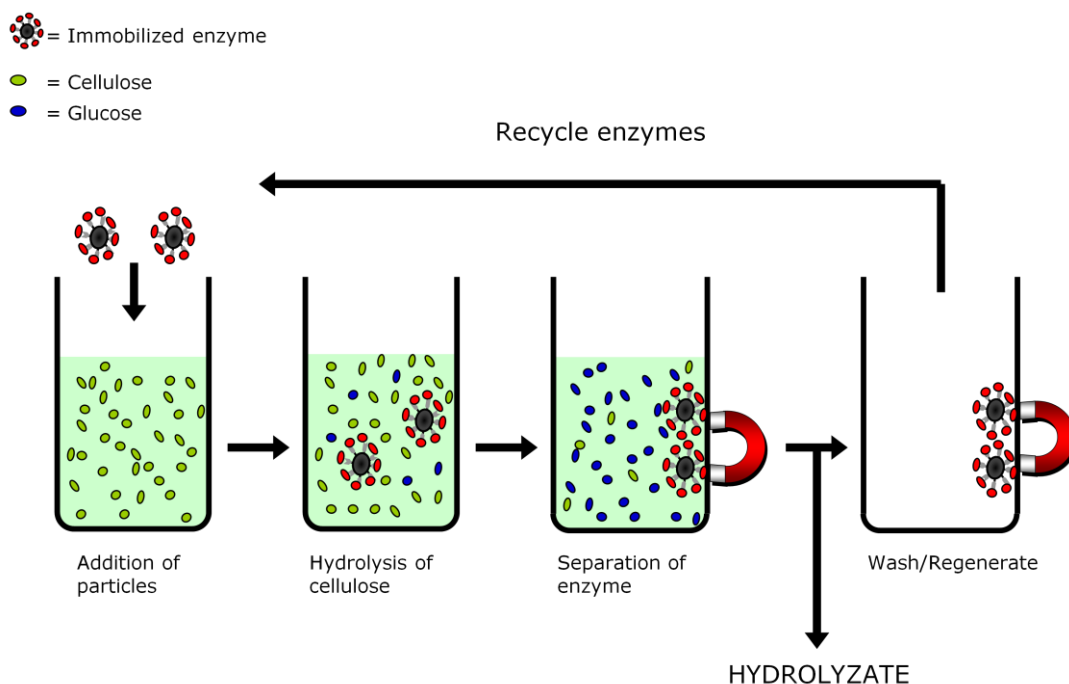## 3. Immobilization of cellulases on magnetic particles

One of the main benefits of enzyme immobilization is the possibility to re-use the enzyme and thereby reducing the production costs of the enzymes. A second advantage commonly observed is increased stability of the immobilized enzyme compared to the free form [39, 40]. During immobilization there are generally three components which should be considered and optimized; enzyme, support and the method of immobilizing the enzyme to the support. For all three components there are numerous possibilities which make the whole procedure of enzyme immobilization a non straightforward action. The enzyme can be immobilized as soluble (using for example ultrafiltration membrane) or insoluble form. The most extensively studied way of immobilization is the latter approach and this could be accomplished by enzyme-support interaction through adsorption, affinity binding, covalent coupling or by gel entrapment of the enzyme [40]. The support material which should be acting as a carrier for the immobilized enzyme should have large surface area, mechanical strength, resistance to microbial attack, many surface groups promoting interaction with the enzyme and should preferably be cheap to produce [41]. One

procedure where no support, or carrier, is used is the formation of cross-linked enzyme aggregates (CLEA) where the enzyme is cross-linked with other enzyme molecules forming an insoluble aggregate [42].

Since the costs of cellulases (representing approximately 0.1€/L produced ethanol [30]) are one of the main obstacles in making the process of lignocellulosic bioethanol economically viable immobilization of the enzymes could be one option aiming to decrease the costs. Recycling of immobilized cellulases using common separation unit operations such as centrifugation or filtration may, however, be difficult when dealing with lignocellulosic feedstocks containing insolubles. One approach to perform recycling in a suspension (containing residual non-hydrolyzed cellulose and lignin) would be to use enzymes immobilized on magnetically susceptible particles [43, 44]. Hence, the immobilized cellulases could be recovered by using a magnetic separator [45] and subsequently re-used for a new cycle of enzymatic hydrolysis of cellulose (Figure 3).

The magnetic particles could be prepared by alkaline precipitation of iron oxides (both Fe(II) and Fe(III)) generating assembled iron-oxide crystal clusters. This is followed by a coating step and the coating layer should be easy to derivatize in order to obtain particles promoting enzyme-particle interaction. The produced particles will possess superparamagnetic properties meaning that they have a high magnetic susceptibility in the presence of an external magnetic field. When the magnetic field is turned off they behave like non-magnetic particles since they have no magnetic memory. Consequently, the particles are easy to re-disperse and could be used for multiple cycles of separation and re-dispersion [45, 46].

Figure 3. Schematic presentation of hydrolysis of cellulose and recycling using magnetically immobilized cellulases.

3.1 Immobilization methods

In this section three different immobilization methods employed in the thesis are described and compared. In paper I and III enzyme was immobilized through covalent coupling between the enzyme and the functionalized magnetic particle. Covalent coupling provides a stable attachment between the enzyme and support giving no or low leakage of the immobilized enzyme. Example of possible reactive residues of the enzyme are amino groups of lysine and N-terminal amino acid, thiol group of cysteine and carboxyl group of aspartate and glutamate. In paper I beta-glucosidase (from Megazyme) was immobilized on non-porous micron-sized superparamagnetic particles activated with different chemistries (activated with cyanuric chloride, polyglutaraldehyde, carboxyl or tosyl groups). The performance of immobilization was evaluated based on bead activity (U/g particles) and the immobilized beta-glucosidase activity was assayed using *p*-nitrophenyl-beta-D-glucopyranoside (PNPG) [37]. It was shown that the highest bead related activities were obtained using particles activated with cyanuric chloride and polyglutaraldehyde (104.7 and 82.2 U/g particles, respectively). These two particle types were used during cellulase immobilization in paper III. The commercial cellulase preparations Celluclast 1.5L and Cellic CTec2 (from Novozymes)

were immobilized and the cellulase activity was assayed using microcrystalline cellulose. It was shown that Cellic CTec2 immobilized on magnetic particles activated with cyanuric chloride gave the highest immobilized cellulase activity (2.8 mg reducing sugar/(g particles*min)). The results in paper I and III confirmed that beta-glucosidase or the whole cellulase mixture could be covalently attached to magnetic particles and retain enzyme activity.

In paper IV ion exchange magnetic particles were used in order to immobilize beta-glucosidase by electrostatic interactions. This is a more simple technique compared to covalent coupling and is therefore generally a cheaper immobilization method. In addition, the particles could be subjected to a stripping (desorbing inactivated enzyme) followed by a re-binding/re-charging step and thus decrease the cost associated with purchase of base particles even more. The main drawback of immobilization through adsorption is the risk of enzyme leakage. Beta-glucosidase (Novozyme 188 from Novozymes) was immobilized on the extremely cheap MIEX$^®$ magnetic particles (referred to as MIEX) and to a Merck magnetic anion exchange particle (referred to as TMAP) of completely different architecture. Both particles are anion exchange particles and the pI of the beta-glucosidase has been reported to be 4.0 [47]. Thus, using an immobilization pH above 4.0 should allow adsorption of beta-glucosidase through ionic binding between enzyme and support. The effect of pH was tested (pH 5.0, 7.0 and 9.0) and it was observed that the highest bead related activities were obtained at pH 5.0 for both MIEX and TMAP particles (30.1 and 67.7 U/g particles, respectively). This was somewhat unexpected since it was thought that increasing pH would result in even more bound beta-glucosidase because of the greater ionization of the enzyme.

The third method of enzyme immobilization tested in this thesis was through affinity binding (employing the very strong biotin-streptavidin system; the interaction between biotin and avidin/streptavidin is one of the strongest found in nature displaying a dissociation constant of 10^{-15} M [48]). In paper II, a beta-glucosidase from *Bacillus licheniformis* was *in vivo* biotinylated in *E. coli* and subsequently immobilized directly from cell lysate on streptavidin coated magnetic particles (optimization trials of beta-glucosidase production are shown in Appendix 1). *In vivo* biotinylation was mediated by fusing the Biotin Acceptor Peptide to the C-terminal of beta-glucosidase and co-expressing the BirA biotin ligase (which promotes efficient biotinylation of the Biotin Acceptor Peptide). This approach enabled simultaneous purification (purification factor of 6.5) and immobilization (3.1 U/g particles) of the enzyme from crude cell lysate on magnetic particles (Figure 4). Generally, both adsorption and covalent immobilization procedures are non-specific, allowing impurities such as other proteins and enzymes to compete for immobilization,

which lowers the specific activity of the particle. On the contrary, affinity binding through biotin-streptavidin interaction eliminates the problem of non-specific binding and in addition to this provides a stable interaction which closely resembles that of a covalent linkage. A third advantage of this method is the possibility of fusing the biotin-tag to the enzyme in a manner so that the catalytic site becomes oriented from the particle (minimizing sterical hindrance). The major drawbacks are the cost associated with derivatization of the particles and the additional work of molecular cloning.
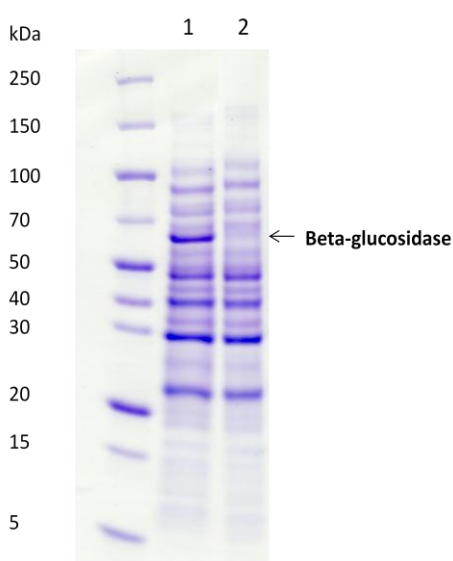
Figure 4. SDS-PAGE of *E. coli* lysate supernatant before and after immobilization of biotinylated beta-glucosidase on streptavidin magnetic particles (paper II). Lane 1, crude enzyme extract prior to immobilization (0.78 mg/ml total protein; 0.013 U/ml free enzyme activity); Lane 2 shows supernatant after immobilization (volume of supernatant was 0.25 ml) using 0.6 mg of streptavidin magnetic particles.

3.2 Specific activity of immobilized enzyme

Specific activity is an important parameter of enzymes and it is determined by units of catalytic activity per mg enzyme/protein (U/mg). During both covalent coupling (paper I and III) and adsorption (paper IV) of the enzyme to the support, the attachment was obtained through a random binding process. The specific activity of an enzyme could therefore decrease upon immobilization because of alterations of the enzyme structure or due to lower accessibility of the substrate to the

active site [49-51]. The latter aspect is most likely more critical for endo- and exoglucanases acting on a large and insoluble substrate (while beta-glucosidase acts on a soluble and small substrate). If the enzyme preparation to be immobilized is not purified the specific activity of immobilized enzyme could be decreased because of preferential attachment of impurities such as other enzymes and proteins. A third possible scenario resulting in lower specific activity is non-favorable micro-environmental conditions. For example, in paper IV anion exchange magnetic particles were used and they could thereby contribute to a micro-environment of a higher apparent pH compared to the bulk.

The specific activity was determined for immobilized beta-glucosidase and whole cellulase mixture in paper I, III and IV and it was observed that in all cases the specific activity decreased upon immobilization. On the contrary, in paper II where biotinylated beta-glucosidase was immobilized on streptavidin coated magnetic particles the specific activity increased by 650% upon immobilization. The increment was observed because of the simultaneous purification step arising from the very high affinity between biotin and streptavidin (Figure 4). In addition to the purification step it was not possible to observe any difference in enzyme activity before and after addition of streptavidin coated magnetic particles to a solution of free biotinylated beta-glucosidase. Thus, the site-directed enzyme immobilization provided unchanged catalytic activity of free and immobilized beta-glucosidase.

3.3 The impact of temperature on the activity of free and immobilized beta-glucosidase

As discussed, enzyme properties could be altered upon immobilization and it is therefore important to examine parameters which could affect the catalytic activity of the enzyme. For the beta-glucosidase (from *Aspergillus niger*) studied in paper I the temperature optimum of free and immobilized enzyme was determined, using the PNPG assay, to 65 and 70ºC, respectively (Figure 5A). This shift in temperature optimum could possibly be explained by an increased thermal stability resulting from immobilization of the beta-glucosidase. During a temperature optimum study two competing aspects exist; increased catalytic activity with increasing temperature and increased enzyme inactivation with increasing temperature. After the optimum temperature is reached, the inactivation effect of temperature is greater than its effect on reaction rate [52]. The thermal stability, or resistance to enzyme inactivation, was studied for free and immobilized beta-glucosidase by incubating the enzyme at 65ºC from 0-5 h and the results are shown in Figure 5B. It

can be observed that after 1 h of incubation, the activity of free and immobilized beta-glucosidase decreased to 40 and 74% of its initial enzyme activity, respectively. After 5 h of incubation the residual activity for free enzyme was close to zero, while there was still activity for immobilized beta-glucosidase (about 36% residual activity). The improved stability for immobilized beta-glucosidase at 65ºC could be due to higher enzyme rigidity upon immobilization making it less susceptibility to enzyme inactivation.
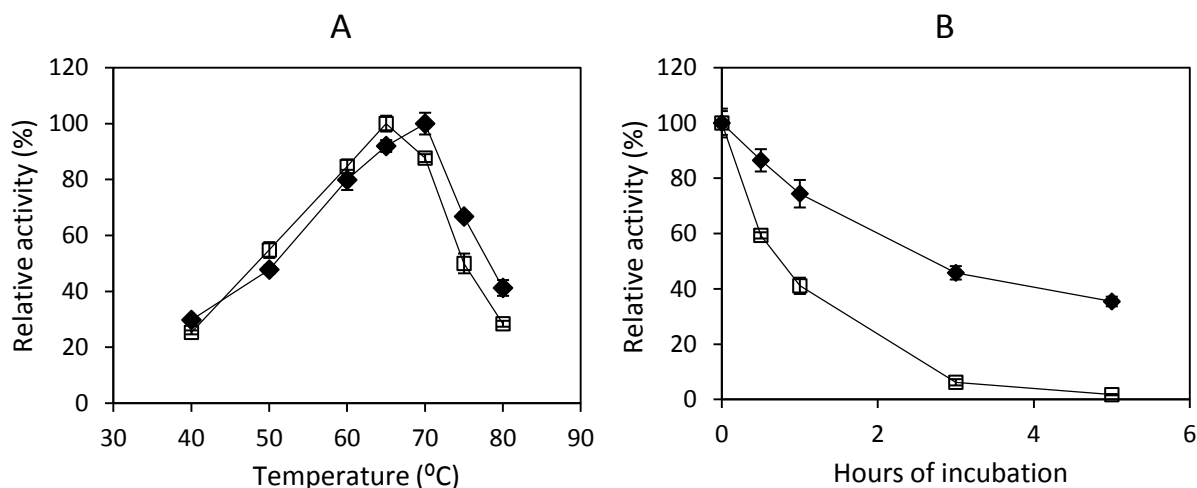
Figure 5. Relative activity for free (□) and immobilized (◆) beta-glucosidase (paper I). A: activity as a function of temperature (within the temperature range of 40-80ºC); B: activity as a function of incubation time. Incubation temperature was 65ºC. The beta-glucosidase activity as assayed using the PNPG assay. Data and error bars represent average and standard deviation, respectively, of 3 replicate experiments.

4. Recyclability of immobilized enzyme

One of the main advantages of enzyme immobilization is the possibility to recycle the enzyme and thereby reducing the manufacturing costs of the enzymes. In all papers included in this thesis the immobilization experiments was therefore accompanied by a recycle study.

4.1 Recyclability of immobilized beta-glucosidase

In paper I the covalent immobilized beta-glucosidase (on particles activated with cyanuric chloride) was combined with free cellulases (Celluclast 1.5L from Novozymes) and utilized for hydrolysis of

bisulphite pretreated spruce. The spruce was composed of % (wt/wt DM): cellulose, 59.3; arabinoxylan, 2.9; galactoglucomannan, 8.3; lignin, 14.3. One hydrolysis cycle was performed for 24 h, at 50ºC, using 8 FPU/g DM spruce and 16 U/g DM spruce of free cellulases (Celluclast 1.5L) and immobilized beta-glucosidase, respectively. It was observed that adding immobilized beta-glucosidase to free cellulases increased the hydrolysis yield of pretreated spruce from 44% to 65%. The results thus confirmed that the immobilized beta-glucosidase could be used on more complex lignocellulosic material (compared to the synthetic soluble PNPG) such as pretreated spruce. After one hydrolysis cycle the immobilized beta-glucosidase was magnetically separated, washed and then used for a new cycle using fresh substrate and Celluclast 1.5L. It was shown that immobilized beta-glucosidase could be used, to boost hydrolysis rate of free Celluclast 1.5L, for at least four hydrolysis cycles. However, it was observed that after the fourth cycle the effect on hydrolysis yield of added immobilized beta-glucosidase had decreased by 52% from the first hydrolysis cycle. The loss in enzyme activity could be due to enzyme denaturation, leakage of attached enzyme (unlikely since the enzyme is covalently attached) or loss of magnetically immobilized enzyme particles during the magnetic separation steps. The loss in activity was investigated in paper II where biotinylated beta-glucosidase immobilized on streptavidin coated magnetic particles was recycled nine times using PNPG as substrate. Experiment A (Figure 6) was performed using a 0.35 Tesla permanent magnet bar and a magnetic separation time of approximately 20 sec. It can be observed that after nine recycles the relative activity had decreased by 54% from the first hydrolysis cycle. The remaining iron content (Fe^{2+} and Fe^{3+} ions) was measured spectrophotometrically [53] and it was concluded that the main contribution to decreased enzyme activity was due to loss of magnetic particles. Therefore, experiment B was performed using a stronger magnet (0.58 Tesla) and an enhanced magnetic separation time (2 min). It can be observed in Figure 6 that when using these conditions the recyclability was improved; after nine recycles the relative activity had decreased by only 11% from the first hydrolysis cycle. Employing these improved magnetic conditions during the enzyme recycling experiment in paper I would probably contributed to an enhanced ability to retain the immobilized enzyme activity.
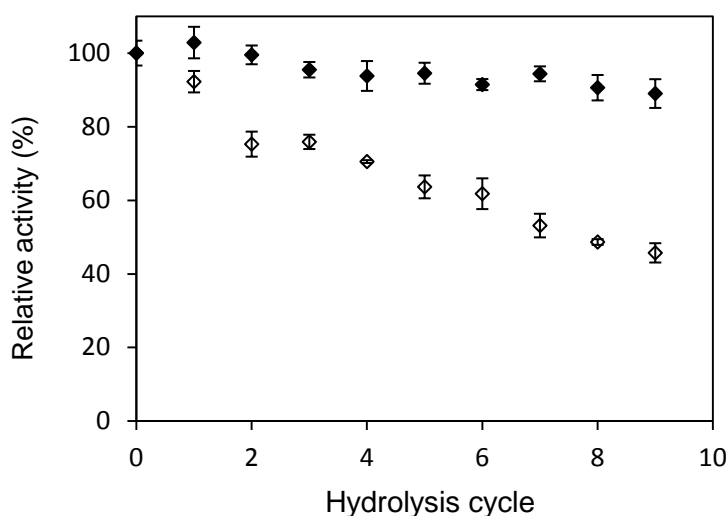
Figure 6. Recyclability study (for a total of nine recycle campaigns) of biotinylated beta-glucosidase immobilized on streptavidin magnetic particles (paper II). One hydrolysis cycle was performed during 30 min at 45ºC using 25 mM PNPG, pH 6.0. Open diamonds, experiment A: magnetic separation was performed using a 0.35 Tesla magnet and 20 sec of separation time; filled diamonds, experiment B: magnetic separation was performed using a 0.58 Tesla magnet and 2 min of separation time. Data and error bars represent average and standard deviation, respectively, of 3 replicate experiments.

In paper IV a similar recyclability study as the one performed in paper I was conducted using immobilized beta-glucosidase on MIEX and TMAP anion magnetic particles. The lignocellulosic substrate was pretreated (hydrothermal) wheat straw composed of % (wt/wt DM): cellulose, 48.1; xylan, 19.5; arabinan, 1.5; lignin, 22.1. One hydrolysis cycle was performed during 20 h at 50ºC using an enzyme loading of 10 FPU/g cellulose of free cellulases (Celluclast 1.5L) and 20 U/g cellulose of immobilized beta-gluocosidase (from Novozyme 188). It was observed that the hydrolysis yield using only Celluclast 1.5L was 46%, while using Celluclast 1.5L with added immobilized beta-glucosidase on TMAP and MIEX particles was 64% and 67%, respectively (Figure 7). Thus, the results confirmed that that immobilized Novozyme 188 could be used to increase the hydrolysis rate of pretreated wheat straw. For the MIEX particles it was possible to re-use the immobilized enzyme and retain activity for at least four hydrolysis cycles. It can be observed that the activity was decreased by 20% between the first and second hydrolysis cycle. However, between the second and the fourth cycle the activity was stable suggesting that loosely

adsorbed beta-glucosidase is leaked during the first cycle while the more strongly attached enzyme is retained throughout the whole hydrolysis campaign. For TMAP particles it was not possible to re-use the enzyme after the first hydrolysis cycle because of interactions with residual insolubles (consisting of non-hydrolyzed cellulose, lignin and hemicellulose) which strongly inhibited separation of the magnetic particles. For the MIEX particles the magnetic separation was very successful since no such interactions occurred and after the fourth hydrolysis cycle the loss of particles was measured and found to be less than 5% (compared to the initial amount).

Comparing the recyclability performance of the particles used in paper I and IV the MIEX particles were definitely the best ones since no interaction between residual insolubles occurred. The worst particles were the TMAP particles because of the substantial interaction with lignin, non-hydrolyzed cellulose and hemicellulose. Another advantage of the MIEX particles was that the particles were rapidly separated (sufficient with 5 sec) while for the other ones tested longer separation times were needed.
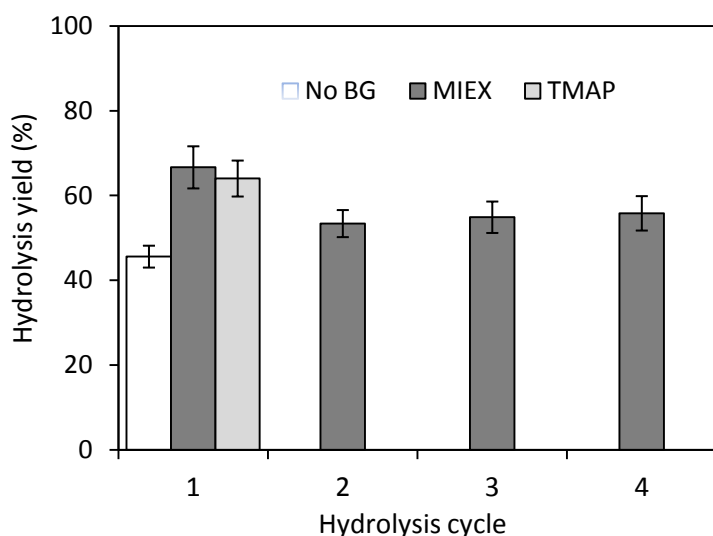
Figure 7. Recyclability of immobilized beta-glucosidase (Novozyme 188) on TMAP and MIEX particles (paper IV). One hydrolysis cycle was performed for 20 h using a WIS content of 2% (w/v) pretreated wheat straw, pH 4.8 (50 mM acetate buffer) at 50ºC. Enzyme activity loading of Celluclast 1.5L and immobilized beta-glucosidase was 10 FPU/g cellulose and 20 U/g cellulose, respectively. No BG (beta-glucosidase) = Celluclast 1.5L only. Data and error bars represent average and standard deviation, respectively, of 3 replicate experiments.

4.2 Recyclability of immobilized cellulase mixture

In paper III a whole cellulase mixture was covalently immobilized on magnetic particles. The most promising approach (Cellic CTec2 immobilized on cyanuric chloride activated particles) was further used for hydrolysis of pretreated wheat straw (composition reported in section 4.1). One hydrolysis cycle was performed during 72 h at 50ºC using 2% water insoluble content (WIS) of pretreated wheat straw and a cellulase loading of 6 FPU/g WIS. The hydrolysis suspension also contained 0.2% bovine serum albumin (BSA). The reason to why BSA was added will be discussed in more detail in section 4.3. One trial was also employed where free Cellic CTec2 was used and the magnetic particles were excluded. After one hydrolysis cycle the immobilized cellulases were magnetically separated, washed and re-used for a second hydrolysis cycle. The results showed that it was possible to hydrolyze pretreated wheat straw using an immobilized cellulase mixture (Figure 8). Furthermore, it was possible to recycle the cellulase mixture and retain activity, although the hydrolysis yield decreased from 82 to 66%.

In paper IV the cellulase mixture Celluclast 1.5L was immobilized on TMAP or MIEX particles and binding capacities of 27.9 and 16.7 mg protein/g particles, respectively, were obtained. The cellulase activity of immobilized enzyme was determined by incubating 9 mg of either the TMAP or MIEX particles (containing immobilized Celluclast 1.5L) with 2% (w/v) microcrystalline cellulose at 50ºC for 24 h. The hydrolysis yield (determined from released glucose) was determined to 46 and 37% for TMAP and MIEX particles, respectively. When the immobilized cellulases were recycled and used for a second hydrolysis cycle the hydrolysis yield was close to zero for both TMAP and MIEX particles. This was most likely due to enzyme desorption from the magnetic particles during the first hydrolysis cycle; endo- and exocellulases consist of a cellulose binding domain which mediates the interaction between the enzyme and cellulose and the high affinity to cellulose promoted desorption of the cellulases from the magnetic particles [33, 34]. In a future study it would be interesting to immobilize cellulases without cellulose binding domains or performing a cross-linking step after adsorption (promoting covalent attachment between enzyme and support).
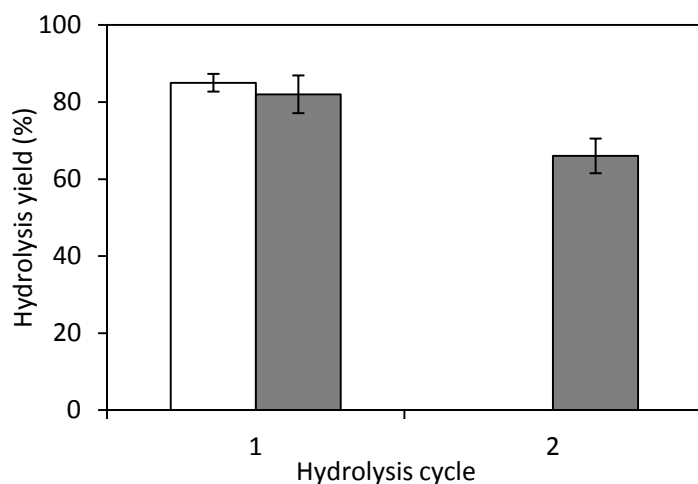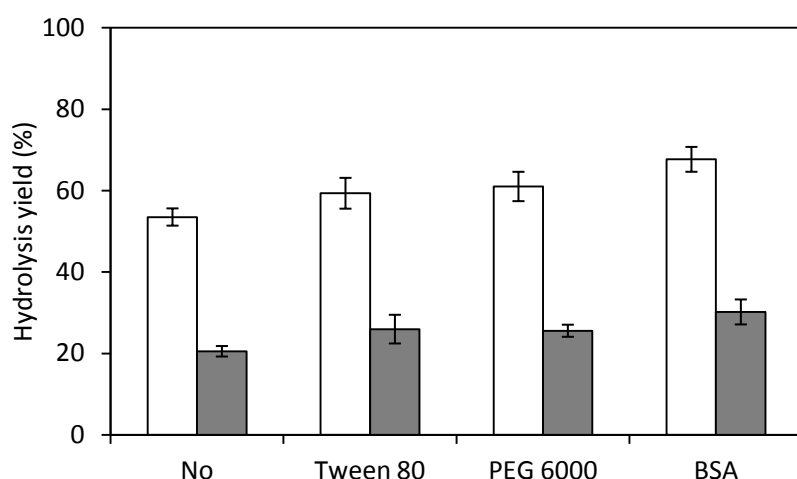
Figure 8. Hydrolysis of pretreated wheat straw using free (unfilled columns) and immobilized (filled columns) Cellic CTec2 (paper III). Cellic CTec2 was immobilized on magnetic particles activated with cyanuric chloride. The water insoluble content (WIS) of pretreated wheat straw was 2% (w/v), suspended in 50 mM acetate buffer (pH 4.8). The added activity of free or immobilized Cellic CTec2 was 6 FPU/g WIS wheat straw and the hydrolysis was performed at 50ºC for 72 h. Data and error bars represent average and standard deviation, respectively, of 3 replicate experiments.

4.3 Effects of surfactants on enzymatic hydrolysis and lignin interaction

Several previous studies have shown that addition of surfactant can increase the hydrolysis rate of cellulose in lignocellulosic biomass [54-56]. This observation is commonly explained by surfactant binding to lignin which reduces the possibilities of unproductive binding between cellulases and lignin. Another recent hypothesis behind enhanced enzymatic hydrolysis of surfactant addition is that surfactants prevent cellulase inactivation induced by cellulose [57]. In paper III, the effect of surfactant addition on cellulose conversion using free or magnetically immobilized cellulases (Cellic CTec2 immobilized on cyanuric chloride activated particles) was examined. The enzymatic hydrolysis was performed using a suspension of 2.5% (w/v) microcrystalline cellulose, 1.25% (w/v) lignin and a surfactant concentration of 0.375% (w/v) of Tween 80, poly(ethylene glycol) (PEG) 6000 or BSA. One experiment was performed as above but excluding surfactant addition. The hydrolysis was performed for 24 h, at 50ºC (pH 4.8) and the cellulase loading of free or immobilized protein was 6.5 mg/g cellulose. It was observed that for both free and immobilized cellulases the presence of surfactants enhanced the enzymatic hydrolysis (Figure 9). The highest

hydrolysis yields of both free and immobilized Cellic CTec2 were obtained using BSA; the yield increased from 54 to 68% and from 21 to 30% for free and immobilized Cellic CTec2, respectively.

During the enzymatic hydrolysis using immobilized cellulases it was observed that the particles interacted with lignin. In order to study this interaction magnetic particles (containing immobilized cellulase) were incubated with lignin and Tween 80, PEG 6000 or BSA. One experiment was also performed where surfactant addition was excluded. The suspension was mixed for 30 min at 50ºC followed by magnetic separation of the magnetic particles. The supernatant was discarded (containing any non-interacting lignin) and the dry weight of residual solids was determined. A reference sample was prepared as described above with the exception that lignin was excluded. The extent of interaction between lignin and the magnetic particles was determined by measuring the increase in dry weight compared to the reference sample. For the experiments using Tween 80, PEG 6000 and when surfactant was excluded the interaction between lignin and magnetic particles was close to 100%; i.e. the weight of the magnetic particles had increased by the weight of added lignin. On the contrary, when adding BSA the separated particles had the same dry weight as the reference sample indicating that no interaction between the particles and lignin had occurred. Thus, addition of BSA yielded the highest enhancement in enzymatic hydrolysis (of the surfactants tested) and inhibited interaction between lignin and magnetic particles. One possible explanation to these two observations could be that decreased interaction between magnetic particles and lignin creates more accessible surface of the magnetic particles containing immobilized cellulases which will in turn increase the possibility of productive interaction between cellulases and cellulose. However, although no decreased interaction between magnetic particles and lignin was observed when using Tween 80 or PEG 6000 the addition of these surfactants improved the hydrolysis yield (Figure 9). This could possibly be attributed to a prevention of cellulase inactivation induced by cellulose as discussed in the paper by Li et al. [57].

Figure 9. The effect of surfactant addition (Tween 80, PEG 6000 and BSA) on hydrolysis yield using free (unfilled columns) and immobilized (filled columns) Cellic CTec2 (paper III). No = no surfactant addition. Hydrolysis was performed for 24 h, at 50ºC (pH 4.8) using a cellulase loading of 6.5 mg/g cellulose of free or immobilized Cellic CTec2. Data and error bars represent average and standard deviation, respectively, of 3 replicate experiments.

5. Scale-up and cost analysis of cellulases immobilized on magnetic particles

5.1 Immobilization and recycling of immobilized beta-glucosidase in pilot scale

In paper I-IV the magnetically recycling of the immobilized enzyme was employed in bench scale using a simple bar magnet. In larger scales similar separation procedure is not applicable and the magnetic particles need to be separated using high gradient magnetic separation (HGMS). HGMS systems consist of a matrix with wires which can become magnetized (high magnetic field gradients around the wires) by an external magnetic field. When the feed, containing magnetic particles, is pumped into the matrix and the external magnetic field is turned on the magnetically susceptible particles will be attracted and captured on the wires [58].

In paper V beta-glucosidase (using the Novozyme 188 preparation) was immobilized on MIEX particles (using similar immobilization conditions as in paper IV) and recycled in pilot scale using a HGMS system. The MIEX particles from Orica Watercare were chosen as support material since they are extremely cheap (they can be supplied in ton quantities for water purification [59, 60]) and the results in paper IV showed very promising properties of enzyme immobilization and recycling. The whole process setup during immobilization and recycling is shown in Figure 10A. The batch

reactor was a 50 L tank with a working volume of 20 L and the reactor was connected to the magnetic enhanced centrifuge (MEC) which captured the magnetic particles. The (MEC) is a HGMS system designed as a centrifuge located inside a strong electromagnet [61, 62]. In the middle of the centrifuge bowl is a matrix of magnetic wires (shown in Figure 10B and C) and with the electromagnet turned on the magnetic wire array creates high magnetic field gradients which enables capturing of the magnetic particles. It is possible to combine magnetically capturing of particles with centrifugation of the bowl. Consequently, the captured magnetic particles on each wire is separated from the wires to the sides of the bowl and by this manner the wire arrays never become saturated with magnetic particles. Centrifugation was not applied in paper V since the amount of particles did not saturate the wires.

Briefly, enzyme immobilization of beta-glucosidase was conducted in the batch reactor using 400 g of MIEX particles (1 h of mixing at room temperature). After immobilization the magnetic particles were pumped into the bowl of the MEC from below (Figure 10A) and the magnetized wires captured the magnetic particles (the electromagnet was turned on) and separated the particles from unbound enzyme. The unbound enzyme was pumped through the central pipe of the MEC (Figure 10A) and directed into a waste container. Then, washing buffer was added into the batch reactor, the electromagnet in the MEC was turned off and the particles were allowed to recirculate in order to wash them. The electromagnet was then again turned on in order to capture the particles and new washing buffer was added for a second step of washing. The capturing and release of the particles containing immobilized beta-glucosidase was highly efficient. After the washing step the magnetically immobilized beta-glucosidase was incubated with microcrystalline cellulose for 20 h at 50°C with mixing. This was performed in order study the performance of the MEC to separate magnetic particles from insoluble cellulosic material. Furthermore, the residual immobilized beta-glucosidase activity was examined in order to determine how much of the beta-glucosidase that had desorbed or been inactivated. After the incubation cycle the magnetic particles were pumped to the MEC and magnetically captured. The capturing of the magnetic particles was highly efficient, though it was more difficult to wash the particles (because of residual cellulose in the wire matrix) compared to the washing after the immobilization step. Five washing cycles (including release, re-circulation and capturing) with 20 L 25 mM acetate buffer were needed to separate all of the cellulose from the immobilized enzyme. The whole procedure of incubation for 20 h, magnetic particle capturing and washing to remove cellulose was performed in total of four campaigns. It was observed that after the first campaign the beta-glucosidase activity was approximately 67% of the

initial activity. This could be due to desorption of loosely bound beta-glucosidase, as observed in paper IV Figure 7. Similar to the results in Figure 7 the beta-glucosidase activity was almost constant between campaign two and four. Subsequent to the fourth campaign the magnetic particles were transferred from the reactor tank to containers for storage. The immobilized enzyme was stored at 4ºC during three months and it was observed that the activity did not decrease upon storage. This demonstrates that although the enzyme was immobilized through adsorption the interaction between the enzyme and support was very strong.
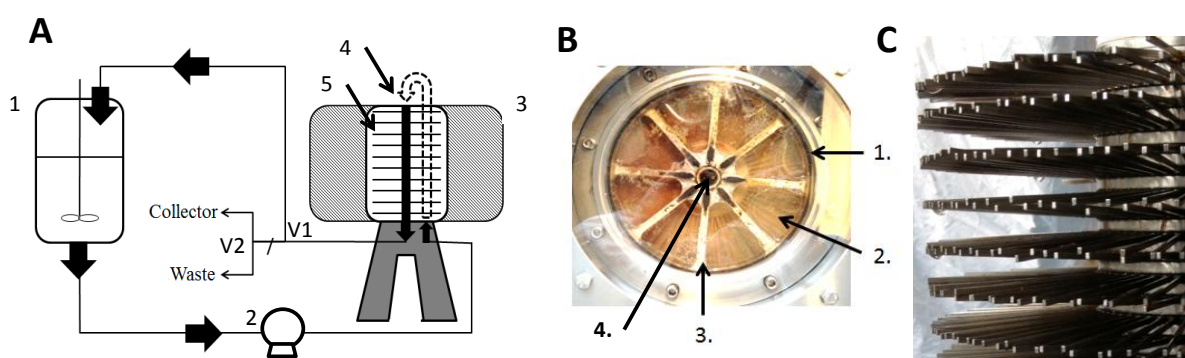
Figure 10. Magnetic centrifuge (MEC) and process setup (paper V). A: illustration of the setup with: 1, batch reactor with overhead mixer; 2, mono pump in recirculation loop with valves V1 and V2; 3, electromagnet of MEC; 4, central pipe for suspension exit from MEC; 5, wire matrix in MEC. B: picture taken through the glass top of the MEC during operation showing: 1, glass lid; 2, rotating wire matrix; 3, glass lid support; 4, central pipe for suspension exit. C: picture taken of the wire matrix located inside the MEC.

5.2 Cost analysis of immobilized cellulases

The cost of the magnetic particles is one of the most important aspects to consider when designing magnetic immobilized cellulases for large scale applications. For example in paper I Novozyme 188 was immobilized on magnetic particles activated with cyanuric chloride and we obtained an immobilized beta-glucosidase activity of 9.8 U/g particles. These commercial magnetic particles are produced for small-scale lab applications and the costs for these (including 80% discount) are approximately 110 €/g particles giving a cost of ca. 11.2 €/Unit of immobilized beta-glucosidase activity (considering materials costs only). On the contrary, for Novozyme 188 immobilized (through adsorption) on the very cheap MIEX particles in paper IV the cost of immobilized beta-

glucosidase activity is estimated to be approximately 3-4 orders of magnitude lower (the costs of the MIEX particles is confidential and exact numbers could therefore not be presented). In addition to the cheap production costs of the MIEX particles the particles could be subjected to a stripping (desorbing inactivated enzyme) followed by a re-binding/re-charging step (see paper IV) and thus decrease the cost associated with purchase of base particles even more. This type of simple re-charging step would not be possible for the particles used in paper I-III since the enzyme is attached through covalent coupling or the very strong affinity binding of biotin and streptavidin.

Below is a simple calculation example of the costs of immobilized cellulases to produce 1 kg of bioethanol and it should be emphasized that many of the numbers are rough assumptions.

- Assume that the concentration of the lignocellulosic biomass used during enzymatic hydrolysis is 160 g/L and that the cellulose content is 50%, i.e. the concentration of cellulose in the suspension is **80 g/L**. Assume that the hydrolysis yield after 72 h (at 50ºC) using immobilized cellulases is **90%**. The glucose concentration in the hydrolyzate is thus:

  0.9 g/g * 80 g/L * 1.11 = **99.9 g/L glucose**

  (where 1.11 is the conversion factor of cellulose to glucose.)

- During fermentation the maximum theoretical yield of ethanol produced from glucose is **0.51 g/g**. Assuming a yield of **90%** of the theoretical yield would thus give a final ethanol concentration of:
  0.51 g/g * 0.9 g/g * 99.9 g/L = **45.9 g/L ethanol**

  (45.9 g/L of ethanol is above the critical limit of 4% which is important for an energy efficient distillation [28].)

- Assume that we need 0.25 gram of particles (containing immobilized cellulases) per gram of cellulose, i.e. the particle concentration is: **20 g/L**

- Assume that the cost of the magnetic particles is: **0.02 €/g particles**

- Cost for free cellulases: **0.13 €/kg ethanol** [30]

- Thus, the costs for producing 1 kg of ethanol using immobilized cellulases is:

  (20 g/L * 0.02 €/g) / 45.9 g/L *1000 + 0.13 €/kg = **8.8 €/kg ethanol**

As can be seen from the calculations above the costs are very high for producing 1 kg of ethanol using cellulases immobilized on magnetic particles. The costs of the free cellulases represent only 1-2% of the costs associated with producing the immobilized enzyme (98-99% of the costs represent the contribution from the particles). However, the benefit of immobilization is the possibility of recycling the cellulases which can be seen in Figure 11A where a simulation of the enzyme costs as a function of number of recycles is shown. It can be seen that after approximately 70 recycles the costs of free and immobilized enzyme is the same. Increasing the number of recycles even more makes the immobilized cellulases more profitable and after 100 hydrolysis cycles the cost of the immobilized enzyme is 0.088 €/kg ethanol; i.e. the cellulase costs is reduced by 32%. The cost simulation in Figure 11 assumes that the immobilized activity remain constant and that the costs of magnetic separation can be neglected (low costs of magnetic separation can be obtained using a permanent magnet in a HGMS system).

In Figure 11B a similar cost simulation is applied, however, in this case only the beta-glucosidase is immobilized and combined with free endo- and exoglucanases. The calculations assume that the cost of beta-glucosidase represent one third of the total enzyme costs and that the amount of magnetic particles could be decreased by a factor of three. It can be seen that the enzyme costs after 100 recycles is 0.116 €/kg ethanol; i.e. the enzyme costs is reduced by 11%. Thus, according to the simulation in Figure 11 the impact on cellulase costs is smaller for immobilized beta-glucosidase compared to when the whole cellulase mixture is immobilized.

In a more detailed survey of cost analysis many more aspects need to be considered such as costs for magnetic separation, washing of particles, lowered specific activity upon enzyme immobilization and the fact that enzyme leakage may occur. In addition, the costs for the enzyme immobilization step have been neglected. However, a potential cheap immobilization approach using the MIEX particles could be performed by combining the immobilization and hydrolysis step. Since the enzyme is immobilized at pH 5.0 and the enzymatic hydrolysis is commonly conducted at the same pH the magnetic particles could just be added to the suspension containing lignocellulose and free enzymes and thereby carrying out a simultaneous hydrolysis and immobilization. In addition, by this manner the unbound enzymes are not going to waste, instead they are directly used (in free form) for hydrolysis.
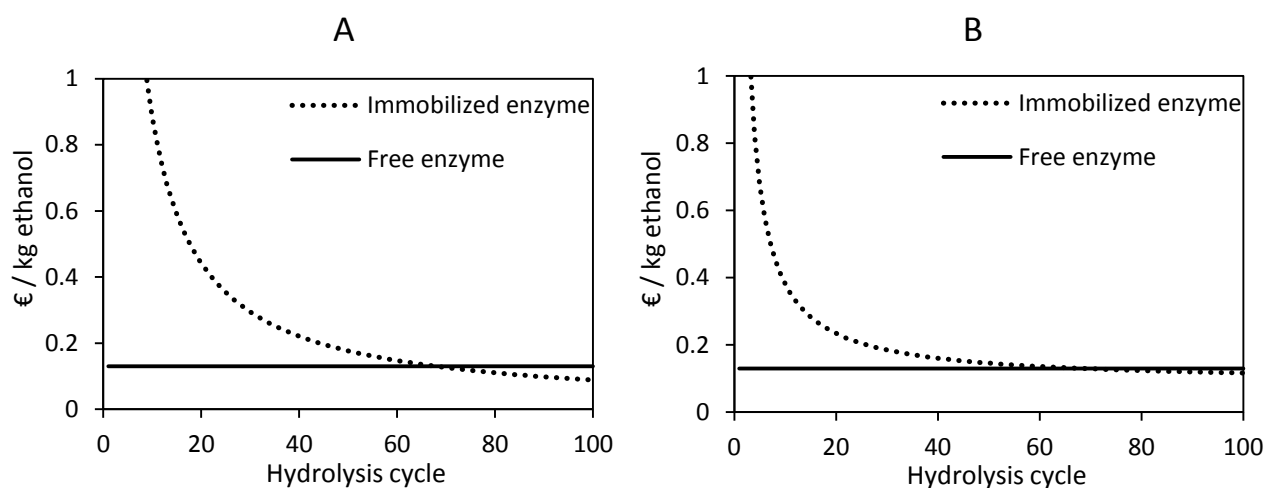
Figure 11. Costs (€/kg ethanol) of immobilized and free cellulases as a function of number of hydrolysis cycles. A: the whole cellulase mixture is immobilized. B: beta-glucosidase is immobilized and combined with free endo- and exoglucanases.

6. Conclusions and future perspectives

The work of this thesis demonstrate that cellulases can be immobilized on magnetic particles and used for hydrolysis of lignocellulosic biomass and subsequently recycled for a new hydrolysis cycle.

The results in paper I and III showed that beta-glucosidase or a whole cellulase mixture (Cellic CTec 2) could be immobilized on magnetic particles through covalent coupling; the highest bead related activities were obtained using magnetic particles activated with cyanuric chloride. Covalent immobilization improved thermal stability of immobilized beta-glucosidase; after 5 hours of incubation, at 65ºC, 36% of activity remained for the immobilized enzyme, whilst there was no activity for the free enzyme.

The hydrolysis yield of cellulose in the presence of lignin was increased when adding surfactants (Tween 80, PEG 6000 or BSA) to magnetically immobilized Cellic CTec2 (BSA yielded the highest increment on cellulose conversion). Furthermore, addition of BSA resulted in a complete inhibition of the interaction between magnetic immobilized cellulases and lignin, while Tween 80 and PEG had no effect.

In paper II, another approach of immobilizing beta-glucosidase was employed where the enzyme was *in vivo* biotinylated and subsequently incubated with streptavidin coated magnetic particles. This enabled simultaneous purification and immobilization from crude cell lysate because of the very strong interaction between biotin and streptavidin.

A third method of enzyme immobilization was performed in paper IV where beta-glucosidase was immobilized through ionic binding using anion exchange magnetic particles. The immobilization was successful and displayed strong interaction between the enzyme and support.

From the different immobilizing studies employed it can be concluded that the most promising particles are the anion exchange magnetic particles from Orica Watercare (MIEX® particles) because of four beneficial attributes: 1) They are extremely cheap and is already now produced in ton quantities for waste water treatment. 2) Even though beta-glucosidase was immobilized through adsorption the interaction was very strong providing low enzyme leakage. 3) They could be rapidly and efficiently magnetically separated from a suspension containing insolubles such as lignin and non-hydrolyzed (surfactant addition were not needed to inhibit the interaction between magnetic

particles and insolubles). 4) The possibility of stripping inactivated enzyme and re-charging with new fresh enzyme could decrease the cost associated with purchase of base particles even more.

The MIEX particles were used in a pilot scale study (20 L scale) and it was possible to efficiently magnetically capture the immobilized enzyme, separate the magnetic particles from residual cellulose and recycle the enzyme for 3 subsequent 20 L incubation cycles. Thus the results in this thesis demonstrate the potential for using cheap magnetic immobilized cellulases in large scale applications such as in the production of lignocellulosic derived biochemicals.

Future studies should focus on hydrolysis of high loadings of lignocellulosic biomass and examine the enzymatic performance using immobilized cellulases. In this thesis the hydrolysis experiments on lignocellulosic substrates has only been conducted using low substrate loadings, however, in an industrial process it is important to obtain high titers of bioethanol and consequently high loadings of lignocellulose are necessary.

In this thesis the enzymatic hydrolysis has been carried out according to the SHF mode where hydrolysis and fermentation is employed separately. However, we are currently conducting a study (results are not included in this thesis) and will continue focus on simultaneous saccharification and fermentation (SSF) using immobilized cellulases on magnetic particles. After one SSF batch the enzymes could be magnetically separated and re-used for a new SSF batch. In addition to the potential of enzyme recycling, immobilization of the cellulases could increase enzyme stability, compared to the free form, and thus make them less susceptible to inactivation from produced ethanol.

Future studies should also focus on enzyme immobilization of endo- and exoglucanases lacking the cellulose binding domain and investigate the potential to cross-link adsorbed cellulases (on MIEX particles) in order to minimize enzyme leakage.

7. Acknowledgments

8. References

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Paper I

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

Johan Alftrén · Timothy John Hobley

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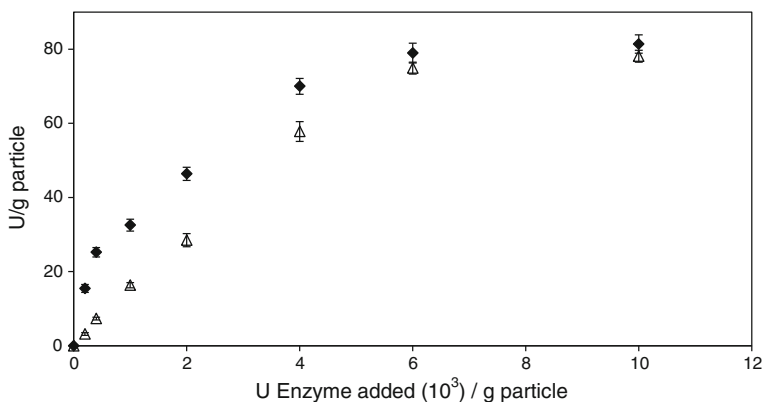
Fig. 1 Bead related activity (U/g particles) after coupling different amounts of Megazyme β-glucosidase to M-Cyanuric (*filled diamonds*) and M-PGL (*open triangles*) particles. Data and *error bars* represent average and standard deviation, respectively, of three replicate experiments

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.
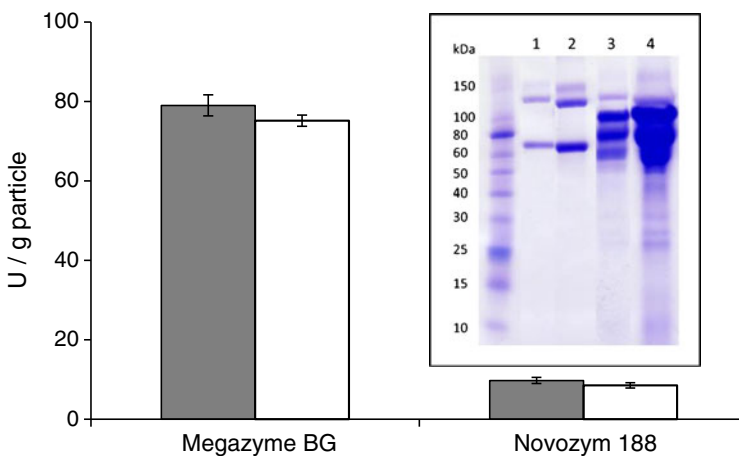
Fig. 2 Bead-related immobilized enzyme activity (U/g particles) when Megazyme β-glucosidase or Novozym 188 have been covalently attached to M-Cyanuric (*filled squares*) or M-PGL (*open squares*) particles. During the immobilization procedure, equal amounts of enzyme units were added (6 U/mg support). 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 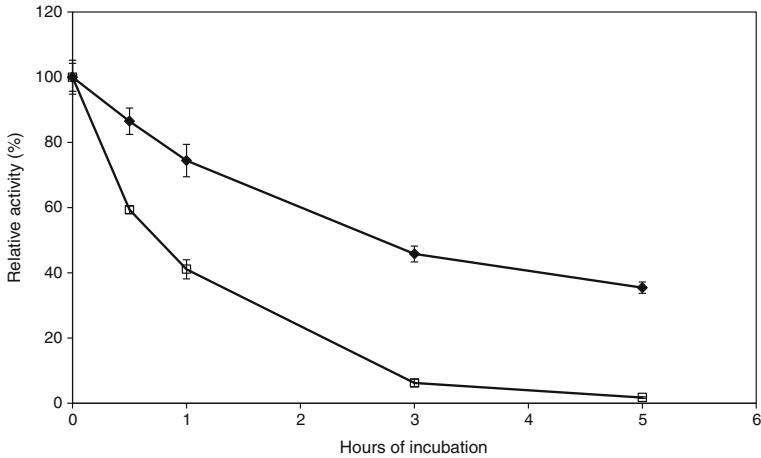and 70 °C for free and immobilized β-glucosidase, respectively). This shift in temperature optimum could possibly be explained by an increased thermal stability resulting from immobilization of the β-glucosidase. Two competing factors exist when performing a temperature optimum study: increased catalytic 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].
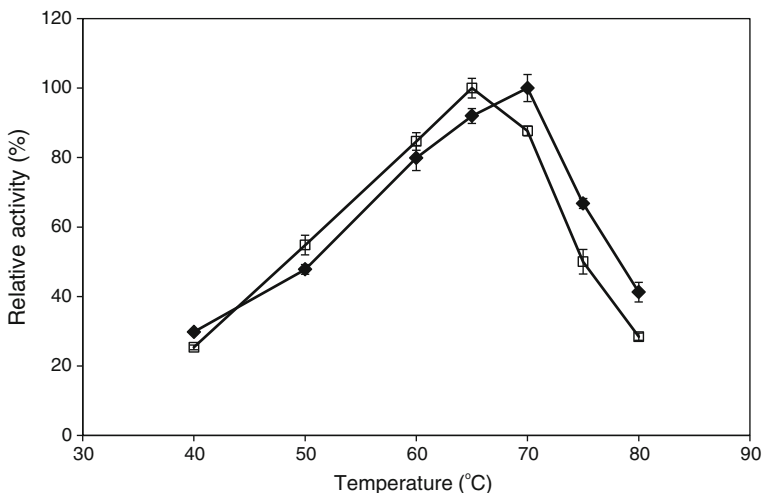
Fig. 3 Activity for free (*open squares*) and immobilized (*filled diamonds*) β-glucosidase as a function of temperature (within the temperature range of 40–80 °C). The maximum activity was normalized to 100 % and in the case of the free and immobilized enzyme were 97.5 U/mg protein and 219.1 U/g particles, 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
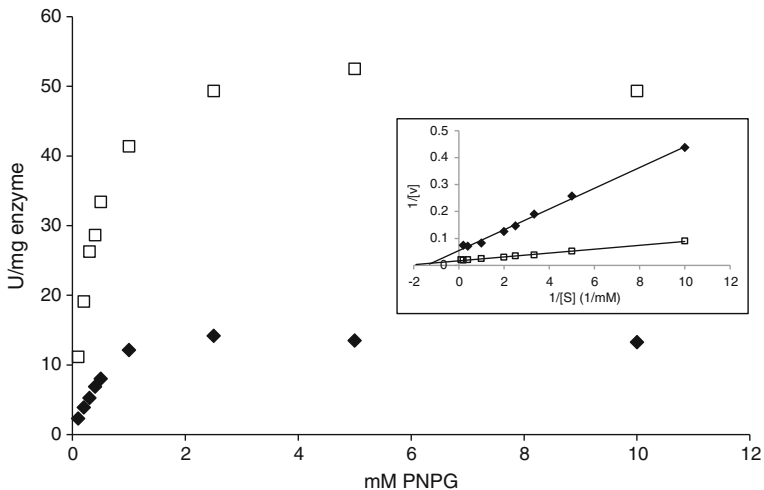

Fig. 5 Michaelis–Menten plot for free (*open squares*) and immobilized (*filled diamonds*) β-glucosidase. The inset shows the Lineweaver–Burk plot of initial hydrolysis rate versus fixed substrate concentration (0.1–10 mM PNPG). Data points are averages of duplicate measurements

(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-
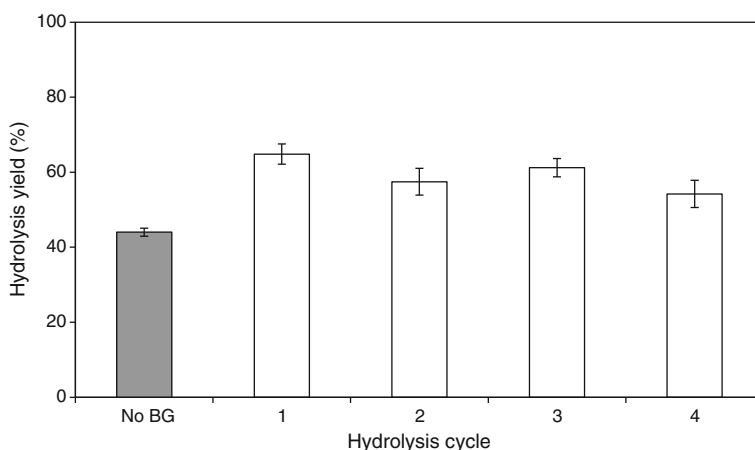
Fig. 6 Hydrolysis yield of pretreated spruce using Celluclast 1.5L in combination with immobilized β-glucosidase (immobilized on M-Cyanuric particles). Hydrolysis yield was determined as liberated reducing sugars divided by the total amount of reducing sugar (total amount of reducing sugar was determined 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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Paper II
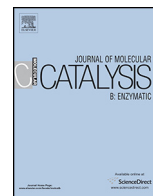
In vivo biotinylation of recombinant beta-glucosidase enables simultaneous purification and immobilization on streptavidin coated magnetic particles

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ABSTRACT

Beta-glucosidase from *Bacillus licheniformis* was *in vivo* biotinylated in *Escherichia coli* and subsequently immobilized directly from cell lysate on streptavidin coated magnetic particles. *In vivo* biotinylation was mediated by fusing the Biotin Acceptor Peptide to the C-terminal of beta-glucosidase and co-expressing the BirA biotin ligase. The approach enabled simultaneous purification and immobilization of the enzyme from crude cell lysate on magnetic particles because of the high affinity and strong interaction between biotin and streptavidin. After immobilization of the biotinylated beta-glucosidase the specific activity (using *p*-nitrophenyl-β-D-glucopyranoside as substrate) was increased 6.5 fold (compared to cell lysate). Immobilization of the enzyme resulted in improved thermal stability compared to free enzyme; after 2 h of incubation (at 50 °C) the residual enzyme activity of immobilized and free beta-glucosidase was 67 and 13%, respectively. The recyclability of immobilized beta-glucosidase was examined and it was observed that the enzyme could be recycled at least 9 times and retain 89% of its initial activity.

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1. Introduction

The non-covalent interaction between biotin and avidin/streptavidin is one of the strongest found in nature displaying a dissociation constant of 10^{-15} M [1]. This property is widely used in different biotechnological applications such as purification [2], immunodetection [3] and immobilization [4]. The term biotinylation refers to a process by which a biotin molecule is covalently attached to specific amino acids on a protein, either chemically or through an enzymatic reaction. Chemical biotinylation suffers the drawback of non-specificity, which can potentially result in structural changes and in an altered enzyme activity profile. The enzymatic approach is mild and highly specific and can be performed both *in vitro* as well as *in vivo*, but requires the presence of a specific biotinylation site on the protein. One of the most widely used biotinylation sites is the Biotin Acceptor Peptide (BAP), which is a short 15 amino acid residues sequence (N′-GLNDIFEAQKIEWHE) containing a single lysine residue to which the biotin molecule can be covalently attached by BirA biotin ligase from *Escherichia coli* [5,6]. Although *E. coli* naturally produces some endogenous BirA biotin ligase it is necessary to express exoge-

nous BirA biotin ligase to obtain an efficient biotinylation [7,8]. Comparing the *in vivo* and *in vitro* procedure, the former has the advantage of performing protein expression and biotinylation simultaneously thereby removing the need for subsequent steps. This can potentially simplify the immobilization of an enzyme to a streptavidin-derivatised support.

Beta-glucosidase hydrolyzes cellobiose to glucose and is an important industrial enzyme used in hydrolysis of cellulosic and lignocellulosic feedstocks [9]. By immobilizing the enzyme to a magnetic particle, operational costs could potentially be reduced due to the possibility of recycling the enzyme through the use of magnetic separation. Immobilization of beta-glucosidase has been reported previously using different support materials and varying attachment methods such as adsorption or covalent reaction between the enzyme and the support [10–16]. Although adsorption is the simplest immobilization method, covalent linkage provides a much more stable attachment, thus minimizing enzyme leakage from the support. However covalent binding may have a negative impact on the catalytic activity due to structural alteration of the immobilized enzyme and/or steric hindrance at the active site [17,18]. Both adsorption and covalent immobilization procedures are generally non-specific, allowing impurities such as other enzymes and proteins to compete for binding, which lowers the specific activity of the support. On the contrary, the biotin-streptavidin system eliminates the problem of non-specific attachment and in addition to this provides a stable interaction which closely resembles that of a covalent linkage. The

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attachment is also directed to a single site on the enzyme, rather than multiple attachment points. In the present work we have *in vivo* biotinylated a heterologously produced beta-glucosidase and subsequently immobilized the expressed enzyme on streptavidin coated magnetic particles. The C-terminal of beta-glucosidase originating from *Bacillus licheniformis* was fused to the BAP-peptide and *in vivo* biotinylated in *E. coli* by co-expressing the BirA biotin ligase. The procedure enables simultaneous purification and immobilization of the enzyme on magnetic particles due to the high affinity and strong interaction between biotin and streptavidin. To the best of our knowledge this is the first study where a beta-glucosidase is *in vivo* biotinylated and subsequently immobilized directly from the cell lysate onto streptavidin coated magnetic particles.

2. Materials and methods

2.1. Strains and plasmids

Bacillus licheniformis DSM 8785 was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). The cloning vector pTwin1 and chemical competent *E. coli* strains DH5α and BL21(DE3) were obtained from New England Biolabs (NEB, USA) (Ipswich, MA, USA). Chemically competent *E. coli* B strain AVB101, harboring plasmid pACYC184 (carrying the *BirA* gene), was obtained from Avidity, LLC (Aurora, Colorado, USA). The GenElute Plasmid Miniprep as well as generic chemicals used in growth media were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Gene amplification and USER cloning

The *bglH* gene encoding a beta-glucosidase was amplified by PCR using a primer pair denoted bglH-F and bglH-R (Table 1). The genomic template was prepared by heating a single *B. licheniformis* colony in 1 ml MilliQ double distilled water for 10 min at 99 °C. The pTwin1 vector was amplified using the primer set designated pTwin1-F and pTwin1-R (Table 1) as well as purified pTwin1 vector as template. PCR amplification was carried out using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) as described by the manufacture using the following procedure. The PCR mixture was incubated at 94 °C for 4 min followed by 35 cycles of 94 °C for 30 s, 62 °C (for *bglH* fragment) or 59 °C (for pTwin1 vector) for 30 s and 72 °C for 2 min. The obtained DNA fragments were verified by agarose gel electrophoresis and purified from the gel using the GFX Purification Kit (GE Healthcare, Buckinghamshire, UK). USER (uracil-specific excision reagent) cloning was performed by incubating 3 μl of the purified *bglH* fragment with 2 μl pTwin1 vector, 2 μl linker (LinkerF and LinkerR), 1 μl USER enzyme mix (NEB, USA), 1 μl NEBuffer4 (NEB, USA) and 1 μl of 1 mg/ml BSA for 20 min at 37 °C, followed by 20 min at 25 °C [19,20]. The linker (Table 1) consisted of a Factor Xa site, the biotinylation site (BAP sequence) and a 6* His tag. It was constructed to generate overhangs complementary to the *bglH* fragment and pTwin1 vector. The final construct is shown in Fig. 1. A 5 μl aliquot of the USER cloning mix was used to transform 50 μl chemically competent *E. coli* DH5α cells (from NEB, USA). Transformed cells were selected on LB (10 g/l tryptone, 5 g/l yeast extract and 10 g/l NaCl)
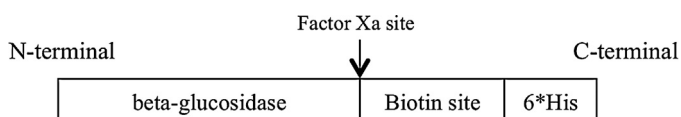
agar plates containing 100 μg/ml ampicillin. Plasmid was recovered from positive clones using GenElute Plasmid Miniprep Kit (Sigma, US), confirmed by restriction analysis and DNA sequencing. The plasmid constructed was termed pTwin1-BglH-BAP.

2.3. Isolation of the BirA encoding vector pACYC184 and transformation in E. coli BL21(DE3) cells

The BirA encoding vector pACYC184 was purified from strain AVB101 and co-transformed together with pTwin1-BglH-BAP into chemically competent *E. coli* strain BL21(DE3) as described by the manufacture. Transformed cells were selected on LB agar plates supplemented with 100 μg/ml ampicillin and 10 μg/ml chloramphenicol. Single colonies were taken and grown overnight in 5 ml LB medium likewise containing 100 μg/ml ampicillin and 10 μg/ml chloramphenicol at 37 °C, with shaking.

2.4. Small scale cultivation conditions

An inoculum of *E. coli* BL21(DE3) cells harboring plasmid pACYC184 and pTwin1-BglH-BAP was prepared and incubated overnight at 37 °C, in LB medium (pH 7.0) supplemented with 100 μg/ml ampicillin and 10 μg/ml chloramphenicol, and if needed 10 mM $MgCl_2$ (discussed later). A 5 ml aliquot of the resulting preculture was subsequently used to inoculate 100 ml LB medium supplemented as previously described, and the resulting mixture was incubated at 37 °C under shaking. When OD_{600} reached 0.7 biotin was added to a final concentration of 50 μM, while IPTG (Sigma–Aldrich, St. Louis, MO, USA) was added to induce expression at a final concentration between 0.05 and 1.0 mM. The beta-glucosidase activity (U/mg total protein) was optimized by varying the IPTG concentration (0.05–1.0 mM) and induction temperature (22–37 °C). The effect of magnesium (on biotinylation) was also examined by varying the concentration between 0 and 10 mM $MgCl_2$. Cells were harvested by centrifugation at $6000 \times g$ for 10 min at 4 °C and lysed by ultrasonication using a MSE 150 Watt Ultrasonic Disintegrator (Measuring & Scientific Equipment Ltd, Crawley, England).

2.4.1. 3-Liter batch fermentation

A 3-L batch fermentation was performed in order to produce a larger amount of recombinant *in vivo* biotinylated beta-glucosidase. The recombinant beta-glucosidase obtained from this batch was used throughout the whole study for enzyme characterization. An overnight inoculum of 30 ml was added to a 5-L fermenter (Biostat B plus, Sartorius Stedim Biotech, Göttingen, Germany) containing 3 L LB medium supplemented with 100 μg/ml ampicillin, 10 μg/ml chloramphenicol, 10 mM $MgCl_2$ and 0.1 ml antifoam. Cultivation was performed using 600 rpm stirring (using two four-bladed Rushton disk turbines), aeration at 3 L/min and a temperature of 37 °C, while pH was maintained at 7. OD_{600} was measured every 30 min and when OD_{600} reached 0.7, filter sterilized IPTG (0.2 mM) and biotin (50 μM) were added and the temperature was decreased to 22 °C (the IPTG concentration and induction temperature were selected from previous optimization trials). After 18 h the cells were harvested by centrifugation at 4 °C at $6000 \times g$ for 10 min. Pellets were resuspended in 0.1 M phosphate buffer, pH 7.4 and disrupted by ultrasonication. Disrupted cells were centrifuged at 4 °C at $12,000 \times g$ for 10 min to remove cell debris and the supernatant was collected and stored at -18 °C until use.

2.5. Purification and immobilization of beta-glucosidase using streptavidin coated magnetic particles

Immobilization of biotinylated recombinant beta-glucosidase was performed using commercially available non-porous

Fig. 1. Schematic presentation of the DNA construct.

Table 1
Primer/linker name and its corresponding sequence.

Primer	Sequence
bglH-F	5′-AGAGCGAUATGACTGAACAAACGAAAAAGTTTCCTG
bglH-R	5′CGCGCCGCUCAAACTCTCGCCATTCGTTTCGATTACTT
pTwin1-F	5′-ATCGCAGAUGATCCGGCTGCTAACAAAGCCC
pTwin1-R	5′-ATCGCTCUTGTATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAG AGGGGAATTG
LinkerF	5′-GGCTCCATTGAAGGCCGCTCCGCGGGCCTGAACGATATTTTTGAAGC GCAGAAAATCGAATGGCATGAAGGCTCCGCGCATCACCATCACCATCACTAATAGATCGCAGAT
LinkerR	5′-CTATTAGTGATGGTGATGGTGATGCGCGGAGCCTTCATGCCATTCGA TTTTCTGCGCTTCAAAAATATCGTTCAGGCCCGCGGAGCGGCCTTCAATGGAGCCCGCGCCGCT

micron-sized (Ø = 1 μm) super paramagnetic particles carrying covalently attached streptavidin (Chemicell GmbH, Berlin, Germany). The particles were washed twice with 0.1 M phosphate buffer (pH 7.4) prior to immobilization. Subsequently 1 mg of particles was mixed with 50 μl cell lysate and 450 μl of 0.1 M phosphate buffer, pH 7.4 at ambient temperature (the protein concentration in the final mixture was 0.39 mg/ml). After 15 min the magnetic particles were magnetically captured, using a bar magnet, and washed twice with phosphate buffer.

2.6. SDS-PAGE

Reducing SDS-PAGE [21] was performed using the Runblue SDS-gel 4–20% system from Expedeon (Cambridgeshire, United Kingdom) as described by the manufacture. The gel was stained by Coomassie Brilliant Blue (CBB R-250, Sigma–Aldrich, St. Louis, MO, USA), destained and subsequently scanned using a CanonScan D660U (Canon Inc., Tokyo, Japan).

2.6.1. Effect of biotinylation

To establish the efficiency of the *in vivo* biotinylated system, excess amounts of avidin (Sigma–Aldrich, St. Louis, Mo, USA) were added to cell lysate and incubated for 15 min (the protein concentration of cell lysate and avidin, in the mixture, was 0.7 and 1.5 mg/ml respectively). Subsequently the mixture was analyzed on SDS-PAGE together with an untreated sample as reference. The resulting band-shift caused by the formation of complexes between the biotinylated beta-glucosidase and avidin served to confirm that an affinity binding interaction did occur.

2.7. Enzyme activity assay

The activity of free beta-glucosidase or immobilized beta-glucosidase (U/g particles) was measured using *p*-nitrophenyl-β-D-glucopyranoside (PNPG; Sigma–Aldrich, St. Louis, Mo, USA), based on a previously described method for free beta-glucosidase [22]. The assay mixture contained 0.9 ml 25 mM PNPG in 50 mM phosphate buffer (pH 6.0) and an appropriate amount of free (between 10 and 100 μl) or immobilized (between 0.5 and 2 mg of magnetic particles) beta-glucosidase in 100 μl phosphate buffer. After incubation at 45 °C for 15 min under gentle shaking, the immobilized enzyme was magnetically separated using a neodymium bar magnet (Supermagnete, Webcraft GmbH, Gottmadingen, Germany). 2 ml 1 M Na$_2$CO$_3$ 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 with a UV-1800 Shimadzu spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD, USA) and a PNP standard curve was used as a reference. The activity measurements were defined as one unit of beta-glucosidase activity (U) releases 1 μmol PNP per min under the assay conditions. The amount of protein attached to the magnetic particles was determined by measuring protein content before and after immobilization in the solution.

In all work conducted, the protein concentration was determined by the Bradford method [23] using bovine serum albumin as standard.

The recombinant beta-glucosidase was treated with protease Factor Xa (Sigma–Aldrich, St. Louis, MO, USA) in order to cleave off the tag consisting of the biotinylation site and 6* His tag. This was performed by incubating cell lysate with Factor Xa (at a mass ratio of 100:1) for 2 h at 30 °C in a 50 mM Tris buffer containing 100 mM NaCl, 6 mM CaCl$_2$, pH 8.0. Subsequently, the protease treatment was analyzed with SDS-PAGE.

2.7.1. Immobilized metal affinity chromatography (IMAC) of free beta-glucosidase

Before characterization of the free biotinylated beta-glucosidase, the enzyme was purified using a HiTrap IMAC FF 1 ml column (GE Healthcare, Uppsala, Sweden) charged with Ni^{2+}. The flow was delivered by a syringe pump (Harvard Syringe pump Type 22, Harvard Apparatus, Holliston, MA, USA) set at 1 ml/min and the flow through was collected using a fraction collector (Helirac 2212, LKB, Bromma, Sweden) set at 0.5 ml/tube. Prior to use the column was equilibrated with 5 column volumes (CV) of equilibration buffer (20 mM sodium phosphate, 0.5 M NaCl, pH 7.4) and subsequently loaded with 10 CV of sample. Then the column was washed with 10 CV of washing buffer (20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, pH 7.4). The bound enzyme was eluted with 5 CV of elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4). The collected fractions were subsequently analyzed for protein content and enzymatic activity as described above.

2.7.2. Characterization of free and immobilized beta-glucosidase

The optimal temperature for free and immobilized beta-glucosidase was determined by performing the PNPG assay at different temperatures within the range of 30–55 °C and the optimal pH was determined at different pH in the range of 4–8, at 45 °C. The thermal stability for free and immobilized beta-glucosidase was examined at 45, 50 and 55 °C. During the thermal stability study, samples were taken at different times over a 3 h period and subsequently assayed according to the PNPG assay as described above.

2.8. Recyclability study of immobilized beta-glucosidase

The ability to recycle magnetic immobilized beta-glucosidase was also examined for 9 sequential 30 min hydrolysis cycles at 45 °C using 25 mM PNPG, pH 6.0. After each cycle the particles were magnetically separated and supernatant was removed, and the separated magnetic particles were washed using 0.1 M phosphate buffer before being recycled. The effect of increasing magnetic force and time of separation was examined (increasing the magnetic force from 0.35 T to 0.58 T and time of separation from 20 s to 2 min). In order to assess if there was any loss of particles during the recycle campaign the iron concentration (both Fe^{2+} and Fe^{3+} ions) was measured spectrophotometrically. Any samples which
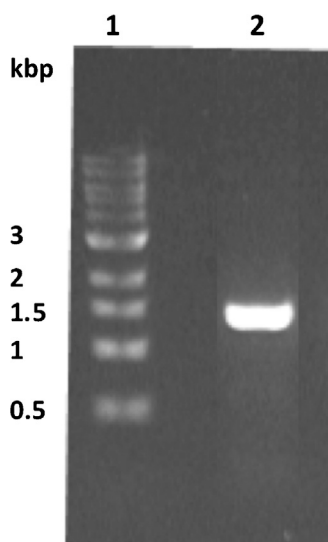
Fig. 2. Agarose gel showing the PCR amplified beta-glucosidase gene *bglH* from *B. licheniformis*. Lane 1, ladder; lane 2, *bglH* gene (1.4 kbp).

may contain particles were subjected to magnetic separation with the bar magnet, and the supernatant was discarded and subsequently any captured magnetic particles were suspended in 0.4 ml 2 M HCl. The suspension was sonicated for 2 h at 40 °C in order to dissolve the particles. Then 40 μl of 10% (w/v) hydroxylammoniumchloride (Sigma–Aldrich, St. Louis, MO, USA) was added and mixed followed by addition of 0.4 ml 0.1% (w/v) 2,2′-bipyridine (Sigma–Aldrich, St. Louis, MO, USA) into the sample. Finally, 0.48 ml of 2 M Tris-(hydroxymethyl)-aminomethane (Sigma–Aldrich, St. Louis, MO, USA) was added to the solution. The absorbance was measured at 522 nm and a standard curve of pure magnetite was used as a reference.

3. Results and discussion

3.1. Expression of recombinant beta-glucosidase and BirA biotin ligase in E. coli BL21(DE3) cells

In this work we have heterologously expressed a beta-glucosidase from *Bacillus licheniformis* in *E. coli.* The expressed beta-glucosidase was *in vivo* biotinylated by fusing a 15 amino acid long peptide (biotin acceptor peptide, BAP) to the enzyme and co-expressing BirA enzyme (biotin ligase). By mixing the expressed beta-glucosidase with streptavidin coated magnetic particles simultaneous purification and immobilization could be achieved due to the high specificity and strong interaction between biotin and streptavidin.

The beta-glucosidase gene *bglH* from *B. licheniformis* was amplified by PCR, according to conditions described above, generating a DNA fragment of approximately 1.4 kb (Fig. 2). The construct in Fig. 1 was generated by USER cloning using the *bglH* fragment, linker and pTwin1 vector. Transformation was performed in *E. coli* DH5α cells and plasmid was recovered from positive clones, confirmed by restriction analysis and DNA sequencing. The plasmid constructed was termed pTwin1-BglH-BAP. In a previous study it is described that the strain AVB101 does not have any T7 RNA polymerase expression system and will therefore not express genes from vectors featuring this system (such as the pTwin1 vector which is used in this study) [24]. Therefore, the BirA encoding vector pACYC184 was purified from strain AVB101 and used for co-transforming it with pTwin1-BglH-BAP into high efficiency chemically competent *E. coli* strain BL21(DE3). Transformed cells

were selected on LB agar plates containing 100 μg/ml ampicillin and 10 μg/ml chloramphenicol.

Expression of beta-glucosidase and BirA biotin ligase was performed by induction with IPTG. Fig. 3A shows an SDS-PAGE of uninduced and induced cells. It can be seen that two protein bands appear after induction (lane 2). The estimated molecular weight of these was 58.5 and 30.6 kDa, corresponding to beta-glucosidase and BirA biotin ligase, respectively. The theoretical molecular weight of beta-glucosidase and BirA biotin ligase is 53.4 and 35.3 kDa, respectively [22,25,26]. When beta-glucosidase was treated with protease Factor Xa to remove the biotin site and 6*His tag the resulting molecular weight was estimated to 54.1 kDa (Fig. 3B), which is close to the theoretical molecular weight of 53.4 kDa.

For uninduced *E. coli* cell cultures no beta-glucosidase activity was detected (based on the PNPG assay), while for induced cells the specific activity of the cell lysate was determined to 0.017 U/mg total protein. In addition to expression of beta-glucosidase from *B. licheniformis* beta-glucosidase from *Aspergillus niger* was also examined for *in vivo* biotinylation in *E. coli.* However, it was neither possible to observe any expression of the enzyme on SDS-PAGE nor to detect any beta-glucosidase activity in cell lysate (data not shown). The reason for unsuccessful expression could possibly be due to codon usage bias.

The efficiency of the *in vivo* biotinylation system was studied by incubating cell lysate with excess avidin. The cell lysate-avidin mixture was analyzed on SDS-PAGE together with an untreated sample as reference. The resulting band-shift caused by the formation of complexes between the biotinylated beta-glucosidase and avidin served to ensure that affinity interaction did indeed occur. Fig. 3C shows the importance of magnesium in order for BirA biotin ligase to catalyze the reaction of biotin ligation. When there was no addition of magnesium in the fermentation medium (lane 1 in Fig. 3C) it can be observed that there is remaining beta-glucosidase (band at 58.5 kDa), which has not formed a complex with avidin. However, when adding 5 and 10 mM of magnesium all the beta-glucosidase has formed a complex with avidin indicating an efficient biotinylation: There is no band at 58.5 kDa in lanes 2 and 3 (Fig. 3C). The effect of magnesium on the catalytic activity of BirA biotin ligase has previously been discussed by Barker and Campbell [5].

3.2. Purification and immobilization of beta-glucosidase on streptavidin coated magnetic particles

Following lysis of the *E. coli* cells and simply mixing with the magnetic particles for 15 min, the beta-glucosidase was efficiently immobilized. The concentration of magnetic particles that was required to bind all the beta-glucosidase was determined by increasing the amount of magnetic particles using a fixed protein concentration (0.78 mg/ml total protein; *i.e.* containing 0.013 U/ml of enzyme activity) of cell lysate. Fig. 4 shows SDS-PAGE analysis of crude enzyme extract, *i.e.* cell lysate, (lane 1) and supernatant after immobilization and magnetic particle removal (lanes 2–8). It can be observed that the immobilization of beta-glucosidase on streptavidin magnetic particles is highly specific. Only the band at 58.5 kDa is removed even when using the highest particle concentration. It can be seen that the beta-glucosidase band completely disappears from lane 3 to lane 4 in Fig. 4, corresponding to a change from using 0.2 mg to 0.4 mg of particles in 0.25 ml, out of the 0.78 mg/ml total protein present before binding, 0.039 mg/ml total protein was bound. The binding capacity under these conditions is thus 24.5 mg/g particles. The SDS-PAGE analysis in Fig. 4 also indicates that the biotinylation of the beta-glucosidase using BirA biotin ligase has been highly efficient. An inefficient biotinylation would result in beta-glucosidase remaining in the supernatant in lanes 5–8 in Fig. 4. In addition, it was not possible to detect any residual beta-glucosidase activity in the supernatant (for lane 5–8).
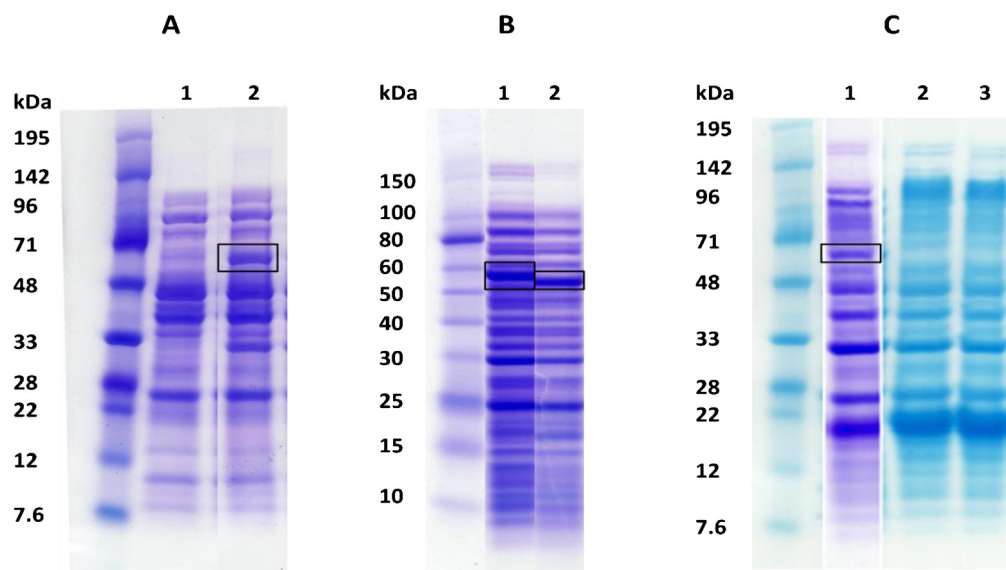
Fig. 3. SDS-PAGE of cell lysate from *E. coli* BL21(DE3) cells harboring plasmid pACYC184 and pTwin1-BglH-BAP. The recombinant beta-glucosidase is indicated with unfilled rectangles. (A) Uninduced (lane 1) and induced cells (lane 2). (B) Effect of treatment with protease Factor Xa: Lane 1, untreated; lane 2, treated with Factor Xa. C, effect of added $MgCl_2$ on biotinylation efficiency: Lane 1, no $MgCl_2$; lane 2, 5 mM $MgCl_2$; lane 3, 10 mM $MgCl_2$. Prior to sample loading the crude enzyme extract was incubated with avidin for 15 min at room temperature.

In order to characterize the free beta-glucosidase the biotinylated beta-glucosidase was purified using a HiTrap IMAC FF 1 ml column. The results in Fig. 5 show one major band (lane 2) of *ca.* 95% purity. In addition, immobilization trials for determining maximum immobilized activity (U/g particles using the PNPG assay) using both cell lysate and IMAC purified enzyme (prior to immobilization) were conducted. The activity of IMAC purified enzyme after immobilization, or enzyme immobilized directly from cell lysate was determined to be 3.1 U/g particles and 2.7 U/g particles, respectively. The difference is most likely attributed to remaining biotin, competing with biotinylated enzyme, in the cell lysate. This problem has been previously discussed in a review on affinity fusion strategies by Nilsson et al. [27]. By removing biotin from the cell lysate (using ultrafiltration with a cut-off of 3 kDa) we obtained similar immobilized activities (*i.e.* 3.2 and 3.1 U/g particles) for cell lysate and IMAC purified enzyme.

The specific activity of beta-glucosidase was determined for cell lysate, IMAC purified and immobilized beta-glucosidase on streptavidin derivatised magnetic particles (Table 2). The specific activity of cell lysate and immobilized beta-glucosidase was determined to 0.017 and 0.11 U/mg, respectively, yielding a purification factor of 6.47. This confirms the observed specificity in Fig. 4 and that simultaneous purification and immobilization was achieved.
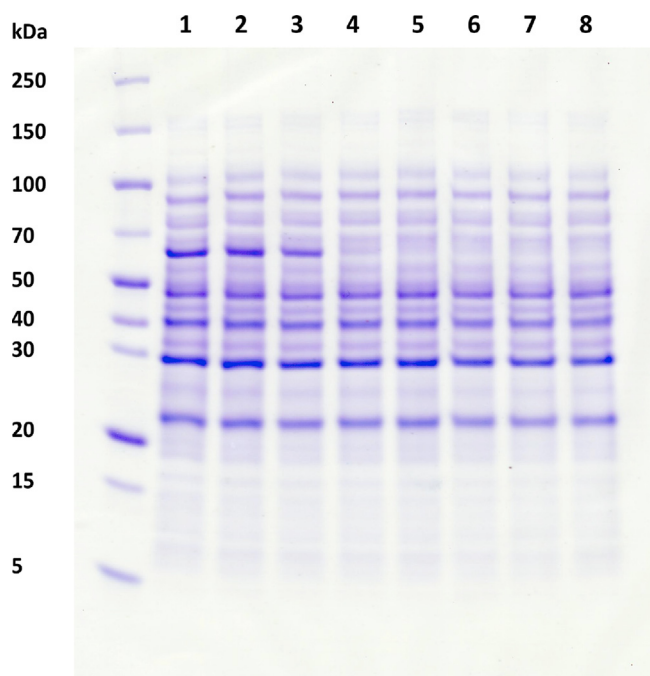
Fig. 4. SDS-PAGE of *E. coli* lysate supernatant after immobilization of biotinylated beta-glucosidase on streptavidin magnetic particles. Lane 1, crude enzyme extract prior to immobilization (0.78 mg/ml total protein; 0.013 U/ml free enzyme activity); Lane 2–8 shows supernatant after immobilization (volume of supernatant was 0.25 ml) using 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 mg of streptavidin magnetic particles, respectively.
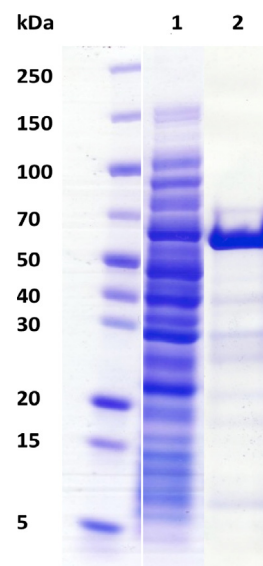
Fig. 5. SDS-PAGE showing the purification of beta-glucosidase using IMAC. Lane 1, induced *E. coli* BL21(DE3) cells harboring plasmid pACYC184 and pTwin1-BglH-BAP. Lane 2, eluate from the IMAC column.
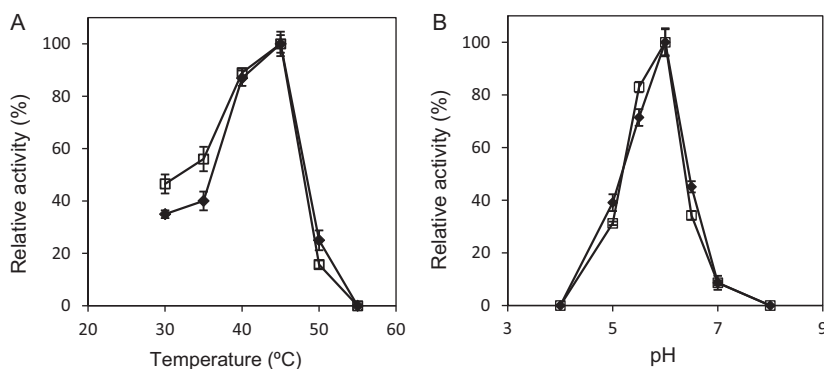
Fig. 6. Optimal temperature (A) and pH (B) for free and immobilized beta-glucosidase determined with the PNPG assay. Legend: free (open squares) and immobilized (filled diamonds) beta-glucosidase (measured within the range of 30–55 °C or pH 4–8). Data and error bars represent average and standard deviation of 3 replicate experiments. The maximum activity was normalized to 100% and in the case of the free and immobilized enzyme were 0.095 U/mg protein and 3.2 U/g particles, respectively.

In order to boost immobilized activity one trial was performed where magnetic particles (non-porous micron-sized ($\varnothing = 1\,\mu m$) super paramagnetic particles) were coated with a higher density of streptavidin (from Chemicell; the binding capacity of these particles, using biotinylated fluorescein, was 30–40% higher compared to low density particles). The maximum bead related activity obtained (using 0.4 mg particles and 1.56 mg/ml protein) was 44% higher (*i.e.* 4.6 U/g particles) using the high density streptavidin particles than those with low density. However, the specific activity decreased (by 12%) compared to when using particles with a lower density of streptavidin.

3.3. Characterization of free and immobilized beta-glucosidase

To compare the optimal temperature for the free and immobilized recombinant beta-glucosidase, enzyme activity was assayed (using PNPG as substrate) within the temperature range of 30–55 °C. In Fig. 6A it can be observed that the optimal temperature for both free and immobilized beta-glucosidase was 45 °C. This is in agreement with findings of Choi et al. [22] expressing beta-glucosidase from *B. licheniformis* and Lun-Cheng and Kung-Ta [28] expressing beta-glucosidase from *Bacillus subtilis*. However, Zahoor et al. [29] obtained an optimal temperature of 50 °C for beta-glucosidase from *B. licheniformis*. Comparing activity as a function of temperature for free and immobilized beta-glucosidase it can be observed that the trend is similar, although the relative activity for free beta-glucosidase at temperatures between 30 and 40 °C is higher compared to immobilized beta-glucosidase. A strong decrease in activity was observed when increasing temperature from 45 to 50 °C which could be explained by enzyme denaturation. At 55 °C the enzyme was completely inactive for both free and immobilized beta-glucosidase.

To compare the optimal pH for free and immobilized beta-glucosidase, enzyme activity was assayed (using PNPG as substrate and 45 °C) within the pH range of 4–8. As shown in Fig. 6B the optimal pH for both free and immobilized beta-glucosidase was determined to be pH 6.0, which is in agreement with the findings

of Choi et al. [22] and Zahoor et al. [29]. It can be observed that the relative enzyme activity for both free and immobilized beta-glucosidase decreases rapidly when decreasing or increasing pH from its optimum. The enzyme was completely inactive at pH 4 and 8.

In order to study thermal stability or resistance to enzyme denaturation, free and immobilized beta-glucosidase was incubated at 45, 50 and 55 °C and the residual activity was determined using standard assay conditions with PNPG. Fig. 7 shows the relative activity as a function of incubation time for free and immobilized beta-glucosidase. It can be observed that both free and immobilized beta-glucosidase are stable at 45 °C for 3 h of incubation. However, at an incubation temperature of 50 °C the relative activity decreases with time and the effect is more pronounced for free beta-glucosidase. After 3 h of incubation the free enzyme is completely inactive while for immobilized beta-glucosidase 57% of its initial activity is retained. The improved stability for immobilized beta-glucosidase at 50 °C could be due to higher enzyme rigidity upon immobilization making it less susceptibility to enzyme denaturation. At 55 °C both free and immobilized beta-glucosidase are completely inactive already after 30 min.

3.4. Recyclability study

One of the advantages with immobilization of enzymes is the possibility of re-using the enzyme which could lower operational costs in a process. Therefore, the potential to recycle the
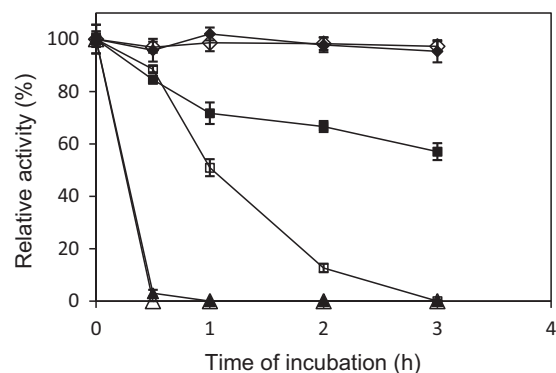
Table 2
Specific activity and purification fold for IMAC purified and immobilized beta-glucosidase on streptavidin magnetic particles. Enzyme activity was determined using the PNPG assay.

Step	Specific activity (U/mg protein)	Purification (fold)
Cell lysate	0.017	na
IMAC purification	0.095	5.59
Immobilized beta-glucosidase	0.11	6.47

na: Not applicable.

Fig. 7. Thermal stability of free (open symbols) and immobilized (closed symbols) beta-glucosidase. Diamonds, beta-glucosidase incubated at 45 °C; squares, beta-glucosidase incubated at 50 °C; triangles, beta-glucosidase incubated at 55 °C. Data and error bars represent average and standard deviation of 3 replicate experiments. The maximum activity was normalized to 100% and in the case of the free and immobilized enzyme were 0.095 U/mg protein and 3.2 U/g particles, respectively.
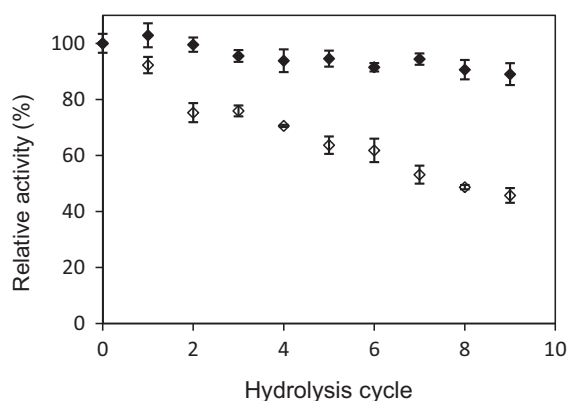
Fig. 8. Recyclability study (for a total of 9 recycle campaigns) of beta-glucosidase immobilized on magnetic particles. Open diamonds, experiment A: magnetic separation was performed using a 0.35 T magnet and 20 s of separation time; filled diamonds, experiment B: magnetic separation was performed using a 0.58 T magnet and 2 min of separation time. Data and error bars represent average and standard deviation of 3 replicate experiments. 100% activity corresponded to 3.2 U/g particles for experiment A and B.

immobilized beta-glucosidase was studied. Fig. 8 shows the relative activity as a function of the number of recycle rounds. Each hydrolysis cycle was performed at 45 °C using 25 mM PNPG, pH 6.0 for 30 min. After one cycle the particles were magnetically separated, washed using 0.1 M phosphate buffer and subsequently used for a second hydrolysis. This was performed in a total number of 9 recycle campaigns. Recycle experiment A was performed using a 0.35 T permanent magnet bar (measured with a Gaussmeter model 410, Lakeshore, Ohio, US) and a magnetic separation time of approximately 20 s. It can be observed that after 9 recycles the relative activity has decreased by 54% from the first hydrolysis cycle. The loss in activity could be due to enzyme denaturation, leakage of attached enzyme or loss of magnetically immobilized enzyme particles during the magnetic separation steps. No leakage of Fe^{2+} and Fe^{3+} ions was observed or any differences in enzyme activity when adding magnetic particles to a solution of free beta-glucosidase. By measuring the remaining iron content (Fe^{2+} and Fe^{3+}) spectrophotometrically it was confirmed that the major contribution to decreased enzyme activity was due to loss of magnetic particles. Hence, experiment B (Fig. 8) was conducted using a stronger magnet (0.58 T) and increased magnetic separation time (2 min). It can be observed that when using these conditions an improvement in recyclability is obtained; after 9 recycles the relative activity has decreased by only 11% from the first hydrolysis cycle.

4. Conclusions

To the best of our knowledge this is the first study where a beta-glucosidase is *in vivo* biotinylated and subsequently immobilized *in situ* in cell lysate on streptavidin coated magnetic particles. The high affinity and strong interaction between biotin and streptavidin enables simultaneous purification and immobilization.

Immobilized beta-glucosidase displays higher thermal stability, at 50 °C, compared to free enzyme. It is possible to recycle the immobilized enzyme at least 9 recycles and retain 89% of its initial activity. The merits of construction of re-useable beta-glucosidase *via* immobilization of (*in vivo*) biotinylated enzyme on streptavidin coated magnetic particles paves the way for studies of immobilizing other enzyme classes, *i.e.* endoglucanases and exoglucanases, relevant for hydrolysis of lignocellulosic feedstocks. Future work should also focus on life cycle analysis of the application of magnetically recyclable enzymes, including modeling of the cost benefits based on pilot scale data.

Acknowledgements

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Paper III

Immobilization of cellulase mixtures on magnetic particles for hydrolysis of lignocellulose and ease of recycling

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ABSTRACT

In the present study whole cellulase mixtures were covalently immobilized on non-porous magnetic particles to enable enzyme re-use. It was shown that Cellic CTec2 immobilized on magnetic particles activated with cyanuric chloride gave the highest bead activity (2.8 mg reducing sugar/(g particles*min)) when using microcrystalline cellulose as substrate. The ratio of the individual activities of endoglucanase, cellobiohydrolase and β-glucosidase changed after immobilization of Cellic CTec2 and it was observed that the specific activity (U/mg protein) decreased upon immobilization. The effect of surfactant addition (using Tween 80, PEG 6000 or bovine serum albumin (BSA)) on hydrolysis yield was studied for free and immobilized Cellic CTec2. It was observed that for both free and immobilized Cellic CTec2 the hydrolysis yield was increased when Tween 80, PEG 6000 or BSA was included. Interaction between magnetic particles (containing immobilized Cellic CTec2) and lignin was examined and it was demonstrated that addition of BSA completely inhibited interaction while Tween 80 and PEG 6000 had no effect on decreasing magnetic particle-lignin interaction. Hydrolysis of pretreated wheat straw was performed in two consecutive cycles using the immobilized cellulases and the results confirmed the potential of hydrolyzing real lignocellulosic substrate and enabling enzyme re-use.

1. Introduction

It is crucial to replace petroleum-based chemicals with more sustainable alternatives since oil is a limited resource and contributes to a net production of greenhouse gases. Lignocellulosic biomass is a potential source for producing environmentally sustainable biobased chemicals, as cellulose is one of the most abundant renewable biopolymers on Earth [1].

An important step for the production of lignocellulosic derived biochemicals is the hydrolysis of cellulose to glucose which can be achieved enzymatically by cellulases consisting of endoglucanases, cellobiohydrolases and β-glucosidases [2, 3]. Endoglucanases randomly hydrolyze internal β-1,4 glycosidic bonds of amorphous cellulose. Cellobiohydrolases cut cellobiose units from the ends of cellulose chains and β-glucosidases hydrolyze cellobiose into two molecules of glucose. One of the major barriers to make the overall bioprocess economically viable is the high cost of the cellulases [4, 5]. Consequently, it is critical to develop new technologies to reduce the amount of enzyme required for the hydrolysis of cellulose. Enzyme immobilization on particles could decrease the enzyme cost by enabling enzyme re-use and increasing enzyme stability [6, 7]. Recycling of free or immobilized cellulases using common separation unit operations such as centrifugation or filtration may, however, be difficult when dealing with lignocellulosic feedstocks containing insolubles. One approach to perform recycling in a suspension (containing non-hydrolyzed cellulose and lignin) would be to use enzymes immobilized on non-porous magnetically susceptible particles [8, 9]. Consequently, the immobilized cellulases could be recovered by using a magnetic separator [10] and subsequently re-used for a new cycle of enzymatic hydrolysis of cellulose.

Immobilization on magnetic particles of either one [8] or all three cellulases has received limited attention and different immobilization methods have been studied, such as covalent attachment [11, 12], adsorption [13] and affinity interaction [14]. Covalent binding and

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affinity interaction generally provides a more stable attachment, minimizing enzyme leakage from the support, compared to adsorption. For adsorptive immobilization of endoglucanases and cellobiohydrolases there is a higher risk of enzyme leakage, compared to β-glucosidases, since they consist of a cellulose binding domain which has a strong affinity for cellulose [15, 16]. Consequently, immobilization of all three cellulases is probably best done through covalent reaction or extremely strong affinity interaction [14].

The objective of the present work was to covalently immobilize commercial cellulase mixtures on non-porous magnetic particles and examine overall immobilized cellulase activity and individual activities of the immobilized enzymes. A second objective was to study the effect of surfactant addition (Tween 80, PEG 6000 or BSA) on enzymatic hydrolysis and interaction between magnetic particles and lignin. Finally, the possibility of using immobilized cellulases for hydrolyzing real lignocellulosic substrate and recycle the enzyme was examined. To the best of our knowledge this is the first study where Cellic CTec2 (a commercial cellulase mixture) is covalently immobilized on magnetic particles. Subsequently, we show it can be used for hydrolysis of pretreated wheat straw. Further, there appear to be no previous studies where the effect of surfactants on the cellulose conversion using magnetic immobilized cellulases has been examined.

2. Materials and methods

2.1. Immobilization of cellulase mixtures on activated magnetic particles

During immobilization of cellulases two different commercial, micron-sized (Ø=1 µm), superparamagnetic particles were studied. They were non-porous silica based and differed primarily in their activation chemistries. They were cyanuric chloride-activated (M-Cyanuric) and polyglutaraldehyde-activated (M-PGL), from Chemicell (Berlin, Germany).

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 5 mg of particles with Celluclast 1.5L or Cellic CTec2 (2.5 FPU added per mg of particles) for 24 h at room temperature, in 1.0 ml 0.1 M phosphate buffer (pH 7.4). Both Celluclast 1.5L and Cellic CTec2 were obtained from Novozymes, Bagsværd, Danmark. The immobilization was stopped by magnetic capture of the particles and washing twice with phosphate 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.

The effect of the amount of Cellic CTec2 added prior to immobilization on immobilized cellulase activity was studied for M-Cyanuric particles. The amount of enzyme added prior to immobilization varied from 0.125 to 3.75 FPU per mg support.

2.2. Measurement of free and immobilized enzyme activity

The cellulase activity (FPU) for free Celluclast 1.5L and Cellic CTec2 was determined by the filter paper assay [17]. For immobilized cellulases a similar procedure, as the filter paper assay, was used for determining cellulase activity. In this case, 0.5 ml of acetate buffer (50 mM, pH 4.8) containing an appropriate amount of immobilized cellulase with 1.0 ml acetate buffer containing 50 mg/ml microcrystalline cellulose powder (Sigma-Aldrich, St. Louis, MO, USA) was mixed and incubated, with shaking, for 1 hour at 50ºC. Subsequently, the tubes were centrifuged at 14000 x g for 30 sec and 0.25 ml of the supernatant was transferred to a new Eppendorf tube containing 0.5 ml DNS (3,5-dinitrosalicylic acid) reagent. The mixture was incubated at 100ºC for 5 min followed by cooling the sample on ice. 0.1 ml of the mixture was then transferred to a cuvette containing 0.9 ml de-ionised water and subsequently spectrophotometrically measured at 540 nm. A standard curve of glucose was

used as a reference.

The endoglucanase activity of free and immobilized cellulase was measured using azo-carboxymethyl cellulose as substrate (Megazyme, Bray, Ireland). 0.2 ml of enzyme solution (free or immobilized) was added to 0.2 ml 20 mg/ml of substrate solution and incubated, with mixing, for 10 min at 50ºC. The reaction was terminated by the addition of 1.0 ml of stop solution (the stop solution was prepared according to instructions from the supplier of the substrate: 40 g sodium acetate trihydrate and 4 g zinc acetate was dissolved in 150 ml water. The pH was adjusted to pH 5.0 with 5 M HCl and the volume adjusted to 200 ml and 800 ml of 96% ethanol was added). The mixture was vigorously stirred for 10 sec using a vortex mixer. Subsequently, centrifugation was conducted for 10 min at 1000 x g and the absorbance of the supernatant was spectrophotometrically measured at 590 nm.

The β-glucosidase activity of free or immobilized Cellic CTec2 was assayed using p-nitrophenyl-β-D-glucopyranoside (PNPG, Sigma-Aldrich, St. Louis, MO, USA), based on a previously described method for free β-glucosidase [18]. 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 enzyme in 100 µl sodium acetate buffer. After incubation at 50ºC for 4 min with gentle mixing, the immobilized enzyme was magnetically separated using a simple ~0.4 Tesla bar magnet. 2 ml 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 min under the assay conditions.

The activity of free and immobilized cellobiohydrolase was assayed using a substrate solution of 1 mM p-nitrophenyl-β-D-cellobioside (Sigma) in acetate buffer, pH 4.8 [19]. The substrate solution contained 1.3 mM D-glucono-1,5-δ-lactone in order to inhibit β-glucosidase from hydrolyzing the substrate. Except for these two differences the assay was performed as

described for the β-glucosidase activity.

The amount of protein attached to the magnetic particles was determined by measuring protein content before and after immobilization in the washing buffer solution. Protein content was estimated by the Bradford method [20] using bovine serum albumin as standard.

2.3. Effect of addition of surfactant on cellulose conversion and lignin interaction

The effect of adding different surfactants on cellulose conversion using free or magnetically immobilized cellulases (Cellic CTec2 immobilized on M-Cyanuric particles) was examined. Enzymatic hydrolysis was performed using a suspension of 2.5% (w/v) microcrystalline cellulose, 1.25% (w/v) lignin (Lignin, organosolv from Sigma) and a surfactant concentration of 0.375% (w/v) of Tween 80, poly(ethylene glycol) (PEG) 6000 or BSA. One experiment was employed as above but without surfactant addition. The cellulase loading of free or immobilized protein was 6.5 mg/g cellulose. The hydrolysis was performed for 24 h, pH 4.8 (50 mM acetate buffer) at 50ºC and glucose formed was measured with HPLC.

Interaction between lignin and magnetic particles was studied by mixing 2 mg of lignin with 5 mg of M-Cyanuric particles (in a total volume of 1 ml) with immobilized Cellic CTec2 in 50 mM acetate buffer, pH 4.8. The suspension contained 0.2 mg/ml of Tween 80, PEG 6000 or BSA. One experiment was also employed with no addition of surfactant. The suspension was mixed for 30 min at 50ºC followed by magnetic separation of the magnetic particles. The supernatant was removed and the dry weight of residual solids was determined by drying the sample at 99ºC for 2 h. A reference sample was prepared as described above with the exception that lignin was excluded. The extent of interaction between magnetic particles and lignin was determined by measuring the increase in dry weight compared to the reference sample.

2.4. Hydrolysis of pretreated wheat straw using immobilized Cellic CTec2

Immobilized Cellic CTec2 on M-Cyanuric particles was used for hydrolyzing hydrothermally pretreated (180ºC for 10 min) wheat straw. The pretreated wheat straw was kindly provided by DTU Risø Campus (Roskilde, Denmark) and the composition as reported by them is given in Table 1. The possibility of recycling the magnetic particles and retaining activity after a hydrolysis cycle was also examined. The trials were performed in 2ml Eppendorf tubes using a water insoluble content (WIS) of 2% (w/v) of pretreated wheat straw suspended in 50 mM acetate buffer (pH 4.8) containing 0.2% (w/v) BSA and 0.05% (w/v) NaN_3 (to prevent microbial growth). One trial was also employed where free Cellic CTec2 was used and the magnetic particles were excluded. The added activity of free or immobilized Cellic CTec2 was 6 FPU/g WIS wheat straw and the hydrolysis was performed at 50ºC with gentle mixing using a vertical rotator (ca 20 rpm). The hydrolysis was terminated after 72 h of incubation by magnetically separating the immobilized Cellic CTec2 using a magnetic bar. The supernatant, containing insolubles, was collected, centrifuged (at 14000 x g for 5 min) and the supernatant from this tube was boiled for 10 min to inactivate any remaining enzyme. The glucose formed was analyzed with HPLC. After one hydrolysis cycle (72 h), the magnetic particles were washed three times with 20 mM phosphate buffer (pH 7.0) containing 0.2% BSA and subsequently used for a second hydrolysis cycle using the same conditions described above with fresh substrate. A reference was prepared using the same hydrolysis conditions but excluding addition of enzymes. The hydrolysis yield was determined by equation 1,

$$\frac{[Glucose]}{1.11 \times f \times [Biomass]} \times 100 = Yield\ (\%) \tag{1}$$

where [*Glucose*] is the glucose concentration (g/L) after a hydrolysis cycle, *f* is the cellulose fraction dry biomass (g/g), [*Biomass*] is the concentration (g/L) of dry biomass at the start of

the hydrolysis and 1.11 is the conversion factor of cellulose to glucose.

Table 1 - Composition of pretreated wheat straw.

Component	% wt/wt DM
Cellulose	48.1
Xylan	19.5
Arabinan	1.5
Lignin	22.1
Ash	4.6

2.5. Analysis of glucose by HPLC

HPLC was used for analyzing glucose formed from hydrolyzed cellulose. Samples were filtered using 0.22 μm filter, transferred to HPLC vials and stored at -20ºC until analysis. The column used was a Hi-Plex K column (Agilent Technologies, Santa Clara, CA, USA) operating at 80ºC and the eluent was MilliQ water using a flow rate of 0.7 ml/min. The glucose formed was detected with a 1260 Infinity refractive index detector (Agilent Technologies, Santa Clara, CA, USA) operating at 35ºC.

3. Results and discussion

3.1. Immobilization of cellulase mixtures on activated magnetic particles

In this study we have covalently immobilized cellulase mixtures on magnetic particles in order to allow enzyme re-use. The overall cellulase activity and individual activities have been studied using two different magnetic particle types. In Table 2 the immobilized cellulase activity is shown for M-Cyanuric and M-PGL particles using the cellulase preparation

Celluclast 1.5L or Cellic CTec2. During immobilization, equal amounts of FPU were added and the cellulase activity of free Celluclast 1.5L and Cellic CTec2 was determined to be 76 and 114 FPU/ml, respectively. The results in Table 2 confirm the hypothesis that cellulase mixtures can be immobilized on magnetic particles and hydrolyze insoluble microcrystalline cellulose. It can be observed that the highest immobilized activity (2.8 mg reducing sugar/(g particles*min)) is obtained using Cellic CTec2 immobilized on M-Cyanuric particles.

The individual enzyme activities of free (U/ml) and immobilized (U/g particles) Cellic CTec2 was determined and the results are shown in Table 3. It can be observed that immobilized endoglucanase and cellobiohydrolase activity using M-PGL particles is 74% and 38% lower, respectively, compared to M-Cyanuric particles. While for β-glucosidase the immobilized activity is 11% higher for M-PGL particles than M-Cyanuric particles. The significant higher activities of endoglucanase and cellobiohydrolase of M-Cyanuric particles compared to M-PGL particles are thus reflected by the higher overall cellulase activity for the former (Table 2).

The amount of protein attached (using Cellic CTec2) to M-Cyanuric and M-PGL particles was 14.6 and 11.9 mg/g particles, respectively. From this it was possible to determine the specific activity (U/mg protein) for the individual immobilized cellulases. Table 4 shows the specific activity for free and immobilized Cellic CTec2 on M-Cyanuric and M-PGL particles. It can be observed that the specific activity is decreased by 50%, 63% and 88% upon immobilization to M-Cyanuric particles for endoglucanase, cellobiohydrolase and β-glucosidase, respectively. Lower specific activity of enzyme immobilized (compared to free) to surfaces and particles other than magnetic types has been reported in previous studies [21-23]. In a recent study, we covalently immobilized a purified β-glucosidase preparation on M-Cyanuric particles and the specific activity decreased by 69% upon immobilization [8]. Xu et al. [12] covalently immobilized a cellulase mixture on magnetic particles activated with

glutaraldehyde and determined enzyme activity of free and immobilized cellulases using soluble carboxymethyl cellulose, rather than the insoluble microcrystalline cellulose used in the current work. They observed a decrease in specific activity of 67% upon immobilization. The loss in enzyme activity could be due to changes of the enzyme structure upon immobilization, non-favorable micro-environmental conditions and lower accessibility of the substrate to the active site for the enzyme which is immobilized in a random orientation. The latter aspect is most likely more critical for endoglucanases and cellobiohydrolases acting on an insoluble substrate (while β-glucosidase acts on a soluble and small substrate).

Table 2 - Cellulase activity (mg released reducing sugar/(g particles*min)) for M-Cyanuric and M-PGL particles immobilized with Celluclast 1.5L or Cellic CTec2. Data represent average of duplicates.

Particle type	Celluclast 1.5L	Cellic CTec2
M-Cyanuric	1.8	2.8
M-PGL	0.7	1.6

Table 3 - Endoglucanase (EG), cellobiohydrolase (CBH) and β-glucosidase (BG) activity for free (U/ml) and immobilized (U/g particles) Cellic CTec2 on M-Cyanuric and M-PGL particles. Data represent average of duplicates.

Enzyme form	EG	CBH	BG
Free Cellic CTec2	3620	74.2	3961
M-Cyanuric	336	6.1	86.0
M-PGL	89	3.8	95.1

Table 4 - Specific activity (U/mg protein) of endoglucanase (EG), cellobiohydrolase (CBH) and β-glucosidase (BG) for free and immobilized Cellic CTec2 on M-Cyanuric and M-PGL particles. The protein concentration of free Cellic CTec2 was determined to be 86 mg/ml. Data represent average of duplicates.

Enzyme form	EG	CBH	BG
Free Cellic CTec2	46.4	0.95	50.8
M-Cyanuric	23.0	0.35	5.9
M-PGL	7.5	0.32	8.0

One trial was also conducted where the effect of smaller particle size (Ø=0.5 µm) on bead activity was examined. Using a particle size of 0.5 µm instead of 1 µm increased bead related activity by 23% (3.4 mg reducing sugar/(g particle*min)) for immobilized Cellic CTec2 on M-Cyanuric particles. The higher activity is most likely due to an increased surface area (by 200% assuming spherical particles) per mass of particles when using magnetic particles with a reduced diameter.

The effect of amount of Cellic CTec2 added prior to immobilization on immobilized cellulase activity was studied for M-Cyanuric particles (in the work until now we have added 2.5×10^3 FPU/g particles). The amount of enzyme added prior to immobilization varied from 0.125 to 3.75 FPU per mg support. Fig. 1 shows that a maximum in immobilized activity is reached at an enzyme addition of 1.25 FPU/mg particles; i.e. adding more enzyme units prior to immobilization will not increase immobilized activity.
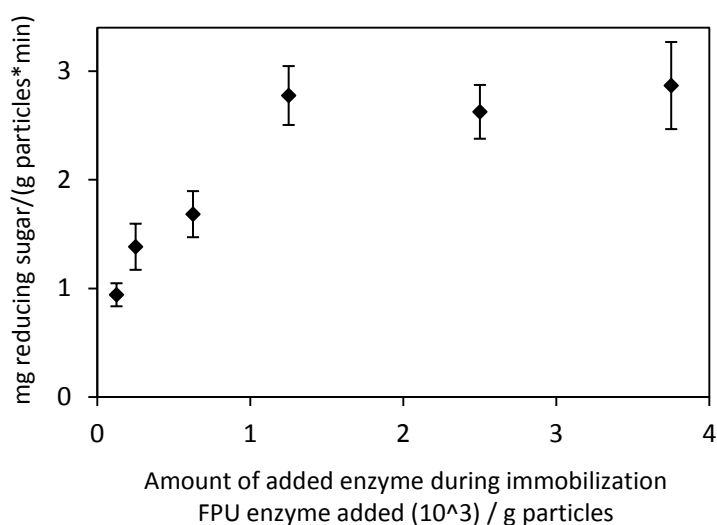
Fig. 1 - Bead related activity (mg released reducing sugar/(g particles*min)) after using different amounts of Cellic CTec2 for coupling to M-Cyanuric particles. Data and error bars represent average and standard deviation, respectively, of 3 replicate experiments.

3.2. Effect of addition of surfactant on cellulose conversion and lignin interaction

The effect of adding different surfactants on cellulose conversion using free or magnetically immobilized cellulases (Cellic CTec2 immobilized on M-Cyanuric particles) was examined. Enzymatic hydrolysis was performed using a suspension of 2.5% (w/v) microcrystalline cellulose, 1.25% (w/v) lignin and a surfactant concentration of 0.375% (w/v) of Tween 80, poly(ethylene glycol) (PEG) 6000 or BSA. (It should be emphasized that we use the term surfactant for PEG 6000 even though it is not fully correct (since it is not an amphiphilic molecule)). One experiment was employed as above but without surfactant addition. The cellulase loading of free or immobilized protein (i.e. enzyme) was 6.5 mg/g cellulose and the hydrolysis was performed for 24 h, pH 4.8 at 50ºC. Fig. 2 shows that when adding similar amounts of enzyme (in mg protein) per gram of cellulose, the free form is more efficient in hydrolyzing cellulose to glucose. This is consistent with the specific activities of the free and immobilized cellulases (Table 4). It can be observed that for both free and immobilized Cellic

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CTec2 the hydrolysis yield is increased when Tween 80, PEG 6000 or BSA is included. It can also be observed that BSA yields the highest improvements in cellulose conversion for both free and immobilized Cellic CTec2 (the hydrolysis yield increased from 54 to 68% and from 21 to 30% for free and immobilized Cellic CTec2, respectively). Kristensen et al. [24] compared the improvement of hydrolyzability of four different surfactants (BSA, Berol 08, PEG 6000 and Twen 80). They used free Celluclast 1.5L in combination with Novozym 188 (both from Novozymes) and they observed highest cellulose conversions with Berol 08 and PEG 6000 addition. A comparison of the results in their study with ours is not fully appropriate since they used another substrate (pretreated wheat straw) and another enzyme formulation compared to the experiments in this study. Nevertheless, the results in our study confirm previous observations on the benefits of surfactant addition and cellulose conversion of lignocellulosic biomass [25, 26]. Further, to the best of our knowledge there are no studies where the effect of surfactants on the cellulose conversion using immobilized cellulases has been examined. The general theory behind increased cellulose conversion of lignocellulosic biomass using surfactants is based on the assumption that the surfactants bind to lignin and thus reduce the possibilities of unproductive binding between cellulases and lignin [27, 28]. However, in a recent study it is suggested that surfactant addition enhances enzymatic hydrolysis due to prevention of cellulase inactivation induced by cellulose [29].
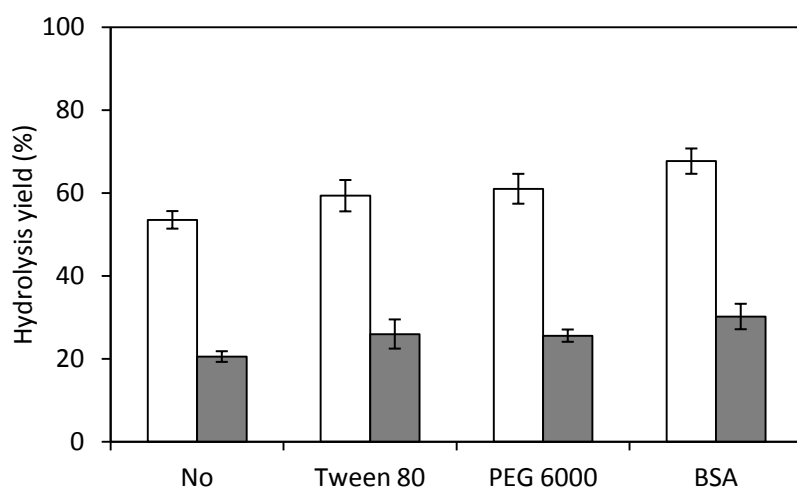
Fig. 2 - The effect of surfactant addition (Tween 80, PEG 6000 and BSA) on hydrolysis yield using free (unfilled columns) and immobilized (filled columns) Cellic CTec2. No = no surfactant addition. Hydrolysis was performed for 24 h, pH 4.8 at 50ºC using a cellulase loading of 6.5 mg/g (i.e. mg free or immobilized protein/g cellulose). Data and error bars represent average and standard deviation, respectively, of 3 replicate experiments.

In order to study the interaction between lignin and magnetic particles, the particles (containing immobilized Cellic CTec2) were incubated with lignin (for 30 min, pH 4.8, at 50ºC) and subsequently the magnetic particles were separated from any non-interacting lignin. A reference sample was prepared as described above with the exception that lignin was excluded. The extent of interaction between magnetic particles and lignin was determined by measuring the increase in dry weight compared to the reference sample. It was observed that when adding BSA the separated particles had the same dry weight as the reference sample, implying that no interaction between the particles and lignin had occurred. On the contrary, for Tween 80, PEG 6000 and when surfactant was excluded, the interaction was close to 100% in all cases; i.e. the weight of the magnetic particles had increased by the weight of added lignin. It is not straightforward to conclude why BSA displayed this unique feature

since the three surfactants tested differ in both size and structure. The molecular weight of Tween 80, PEG 6000 and BSA are 1.3, 6.0 and 67 kDa, respectively. Thus, the size of BSA is considerably larger than the two other surfactants tested and could possibly promote more sterical hindrance when attached to lignin and in turn prevent interaction with the magnetic particles. Lignin has a hydrophobic surface and BSA is well known to be a soft protein that interacts non-specifically via hydrophobic interactions to many surfaces. It is interesting to emphasize that BSA, which fully inhibited magnetic particle-lignin interaction, gave the highest improvements in hydrolysis yield (Fig. 2). Reducing the magnetic particle-lignin interaction will create more accessible surface of the magnetic particles containing immobilized cellulases which will increase the chance of productive interaction between the cellulases and cellulose. However, although no reduced magnetic particle-lignin interaction was observed when using PEG 6000 or Tween 80 the addition of these chemicals enhanced the hydrolysis yield (Fig. 2). This observation could possibly be attributed to a prevention of cellulase inactivation induced by cellulose as discussed in a recent paper by Li et al. [29].

3.3. Hydrolysis of pretreated wheat straw using immobilized Cellic CTec2

Immobilized Cellic CTec2 (on M-Cyanuric particles) was used for hydrolyzing hydrothermally pretreated wheat straw (the composition is reported in Table 1). The possibility of recycling the magnetic particles and retaining activity after one hydrolysis cycle was also examined. The trials were performed in 2ml Eppendorf tubes using a water insoluble content (WIS) of 2% (w/v) of pretreated wheat straw suspended in 50 mM acetate buffer (pH 4.8) containing 0.2% BSA (w/v) and 0.05% (w/v) NaN_3. One trial was also employed where free Cellic CTec2 was used and the magnetic particles were excluded. The added activity of free or immobilized Cellic CTec2 was 6 FPU/g WIS wheat straw and the hydrolysis was performed at 50ºC for 72 h. Fig. 3 shows the hydrolysis yield for free and immobilized Cellic

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CTec2 and the yield was determined to be 85 and 82%, respectively, during the first use. This confirms the possibilities of using the immobilized Cellic CTec2 for hydrolysis of real lignocellulosic feedstock. Subsequent to the hydrolysis cycle the magnetic particles were magnetically separated, washed and used for a second hydrolysis cycle. It can be observed that after one recycle the hydrolysis yield has decreased from 82% to 66%. Although there is a decrease in cellulose conversion (after one hydrolysis cycle) the results show the potential of reducing the enzyme amount required, during hydrolysis, by using magnetically immobilized cellulases. This confirms and extends our previous studies showing that magnetic immobilized β-glucosidase could be used for multiple cycles in pretretreated spruce [8, 14].
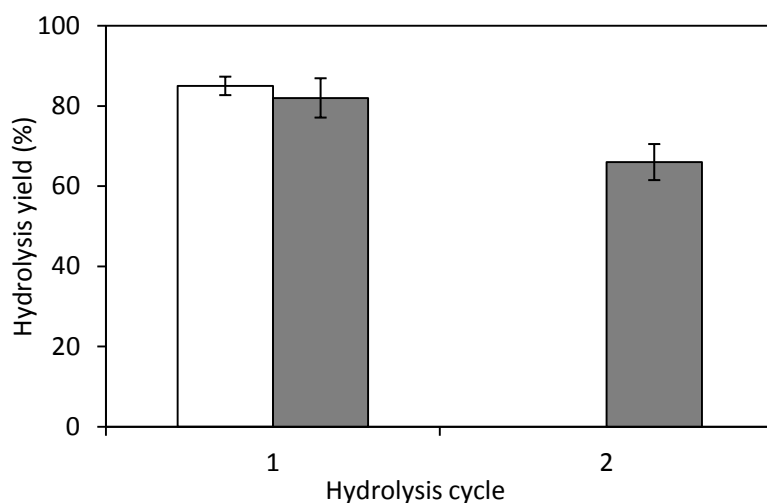
Fig. 3 - Hydrolysis of pretreated wheat straw using free (unfilled columns) and immobilized (filled columns) Cellic CTec2. Cellic CTec2 was immobilized on M-Cyanuric particles. The water insoluble content (WIS) of pretreated wheat straw was 2% (w/v), suspended in 50 mM acetate buffer (pH 4.8). The added activity of free or immobilized Cellic CTec2 was 6 FPU/g WIS wheat straw and the hydrolysis was performed at 50ºC for 72 h. Data and error bars represent average and standard deviation, respectively, of 3 replicate experiments.

4. Conclusion

To the best of our knowledge this is the first study to show that Cellic CTec2 can be covalently immobilized on non-porous magnetic particles. The hydrolysis yield of cellulose in the presence of lignin is increased when adding surfactants (Tween 80, PEG 6000 or BSA) for both free and magnetically immobilized Cellic CTec2 (BSA gives the highest increment on the hydrolysis yield). Further, addition of BSA results in a complete inhibition of the interaction between magnetic immobilized cellulases and lignin, while Tween 80 and PEG have no effect. It is possible to hydrolyze real lignocellulosic biomass (pretreated wheat straw) using immobilized cellulases and the results in this study confirm the potential of re-using magnetically immobilized cellulases for consecutive hydrolysis cycles.

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Paper IV

Immobilization of beta-glucosidase by adsorption on anion exchange magnetic particles enables enzyme re-use during hydrolysis of pretreated wheat straw

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Abstract

Cheap and robust immobilization of enzymes to magnetic particles may be a cost effective solution for pretreatment of lignocellulosic materials. In the present study beta-glucosidase (Novozyme 188) which hydrolyzes cellobiose was immobilized, via simple adsorption, on two different types of anion exchange magnetic particles (TMAP and MIEX) in order to allow re-use of the enzyme. pH was varied during immobilization (pH 5.0, 7.0 and 9.0) and it was observed that pH 5.0 yielded highest immobilized enzyme activity; 67.7 and 30.1 U/g particles for TMAP and MIEX particles, respectively. It was not possible to strip the immobilized enzyme under conditions akin to those in lignocellulose hydrolysis and it was possible to strip the enzyme only using extreme conditions (1 M NaCl, pH 3.0). The stripped particles could be successfully used for re-binding of fresh enzyme. A complete cellulose mixture (Celluclast 1.5L) could also be immobilized; however in that case the cellulases were desorbed after one use. Immobilized beta-glucosidase was combined with free cellulases and used for hydrolysis of pretreated wheat straw. The results confirmed that beta-glucosidase immobilized on anion exchange magnetic particles could be used to boost cellulose hydrolysis (by 46%) on real lignocellulosic substrate. Further, it was possible to re-use the immobilized enzyme and retain activity for four consecutive hydrolysis cycles.

Keywords: Enzyme immobilization, cellulases, biomass, biorefinery, bioethanol

1. Introduction

Lignocellulosic biomass is a potential source for producing environmentally sustainable biobased chemicals, given that cellulose is one of the most abundant renewable biopolymers on Earth (Schurz, 1999). An essential step for the production of chemicals derived from lignocellulose is the enzymatic hydrolysis of cellulose to glucose which can be obtained by the synergistic action of endoglucanases, exoglucanases and beta-glucosidases (Bommarius et al., 2008; van Dyk and Pletschke, 2012). Endoglucanases hydrolyze internal beta-1,4 glycosidic bonds of amorphous cellulose. Exoglucanases cleave off cellobiose units (dimers of glucose) from the ends of cellulose chains and beta-glucosidases hydrolyze cellobiose into two molecules of glucose. The cost of enzymes (cellulases) is of one of the major bottlenecks in the overall bioprocess and it is thus essential to develop new enzyme technologies to make the process more economically feasible. Immobilization of either one or all three cellulases on particles could reduce the enzyme cost by improving operational stability of the enzyme and allowing re-use (Mateo et al., 2007; Sheldon, 2007). However, recycle of enzymes using common separation unit operations such as filtration or centrifugation is difficult when handling lignocellulosic feedstocks containing insolubles. One way to overcome the difficulty in recycling would be to use enzymes immobilized on magnetically susceptible particles (Alftrén and Hobley, 2013; Koneracká et al., 2006; Schultz et al., 2007). The immobilized enzymes could thus be magnetically separated by applying an external magnetic field and subsequently re-used for several cycles of hydrolysis.

Enzyme immobilization on magnetic particles has previously been reported using varying attachment methods such as adsorption (Khoshnevisan et al., 2011; Lupoi and Smith, 2011), covalent coupling (Dekker, 1990; Jordan et al., 2011; Xu et al., 2011) and affinity binding (employing the very strong biotin-streptavidin system) (Alftrén et al., 2013). Covalent coupling normally offers a more stable attachment, reducing the risk of enzyme leakage from

the support, compared to adsorption. However, this is costly and large amounts of enzyme are not active after immobilization (Alftrén and Hobley, 2013). Furthermore, immobilized enzyme will be gradually inactivated and it would be advantageous if it could be removed to allow the magnetic base particle to be re-used for manufacturing new immobilized enzyme. Employing simple adsorption as the immobilization method could lead to extremely cheap immobilized enzymes. Magnetic anion exchangers such as MIEX® from Orica (Orica Watercare, Australia: http://www.miexresin.com/) can be produced so cheaply that they can be supplied in ton quantities for water purification (Mergen et al., 2008; Singer and Bilyk, 2002). Furthermore, the particles could potentially be subjected to a stripping (desorbing inactivated enzyme) followed by a re-binding/re-charging step and thus decrease the cost associated with purchase of base particles even more. In the present work we have examined the potential of the extremely cheap MIEX® magnetic particles for immobilization of beta-glucosidase (Novozyme 188 from Novozymes), by simple adsorption, and compared them to a Merck magnetic anion exchange particle of completely different architecture, which is available in kilogram amounts. Immobilization conditions, re-use of base particles and the possibility of recycling the immobilized beta-glucosidase during use on real lignocellulosic substrate have been examined. To the best of our knowledge this is the first study where MIEX® or TMAP magnetic particles have been examined for enzyme immobilization. Furthermore, there have been no previous studies where magnetic particles have been re-charged subsequent to an initial immobilization procedure.

2. Materials and methods

2.1. Immobilization of Novozyme 188 on anion exchange magnetic particles

In this study immobilization of Novozyme 188 has been performed using two different types of magnetic adsorbents; MagPrep TMAP 100 developed by Merck KGaA, Darmstadt,

Germany and MIEX® from Orica Watercare, Melbourne, Australia. The MagPrep TMAP 100 magnetic particles (referred to as TMAP) are non-porous beads with a size of 100 nm that are produced by magnetite precipitation, silica coating and functionalized with tri-methyl-ammonium-propyl (TMAP) tentacles up to 30 monomers long functioning as strong anion exchangers (personal communication: Karl Holschuh, Merck, Darmstadt, Germany). The magnetic ion exchange bead MIEX® from Orica Watercare (Ballard et al., 2001; Mergen et al., 2008; Singer and Bilyk, 2002) are macroporous with a diameter of 150-180 μm. The bead functions as a strong anion exchanger with quaternary amine functional groups. These particles will be referred to as MIEX.

Prior to enzyme immobilization the particles were first equilibrated for 10 min with 20 mM phosphate buffer (pH 7.0) containing 0.5 M sodium sulphate (~10 mg particles/mL buffer) followed by a washing step with 20 mM phosphate buffer (pH 7.0) (~10 mg particles/mL buffer) to remove excess ions. Enzyme immobilization was performed in Eppendorf tubes by mixing particles (either TMAP or MIEX) with Novozyme 188 (Novozymes, Bagsværd, Danmark) at a concentration of 0.1 mg protein Novozyme 188 per mg particles for 30 min with shaking (850 rpm) at room temperature. The pH before binding was adjusted to 5.0, 7.0 or 9.0 with NaOH and the final concentration of protein was approximately 1 mg/mL in all trials. After 30 min, the immobilization was terminated by magnetic capture of the particles and they were washed with 20 mM phosphate buffer (pH 7.0). Additional experiments were conducted where immobilization time was varied between 5 sec - 6 h.

The possibility of immobilizing a beta-glucosidase preparation (Accellerase BG) from Dupont Industrial Biosciences (Palo Alto, CA, USA) was also examined. The immobilization on TMAP and MIEX particles was performed as described above.

2.2. Enzyme assay and protein determination of free and immobilized beta-glucosidase

The activity of free or immobilized beta-glucosidase (U/g particle) was assayed using *p*-nitrophenyl-beta-D-glucopyranoside, PNPG (Sigma-Aldrich, St. Louis, MO, USA), based on a previously described method for free beta-glucosidase (Berghem and Pettersson, 1974). The assay mixture contained 0.9 mL of 5 mM PNPG in 50 mM sodium acetate buffer (pH 4.8) and an appropriate amount of free (between 10-100 µl) or immobilized Novozyme 188 (between 0.5-1.0 mg particles) in 100 µl sodium acetate buffer was added to give a final volume of 1 mL. After incubation at 50ºC for 5 min with mixing (850 rpm), the immobilized enzyme was magnetically separated using a simple ~0.4 Tesla bar magnet. 2 mL 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 beta-glucosidase activity (U) releases 1 µmol PNP per min under the assay conditions.

The binding capacity (amount of bound protein) of the magnetic particles was determined by measuring protein content of the Novozyme 188 solution before and after immobilization and in the washing fraction. Protein content was estimated by the Bradford method (Bradford, 1976) using bovine serum albumin as standard within the concentration range of 0-1 mg/mL.

2.3. The effect of binding pH on enzyme recyclability

The effect of binding pH (pH 5.0, 7.0 and 9.0) on recyclability of immobilized Novozyme 188 on TMAP and MIEX was assessed for four sequential 5 minute hydrolysis cycles at 50ºC using 5 mM PNPG, pH 4.8. After each cycle the particles were magnetically separated and the supernatant was removed. The separated magnetic particles were washed twice with 20 mM phosphate buffer (pH 7.0) before being recycled.

2.4. Stripping and re-binding of Novozyme 188 on TMAP and MIEX particles

The ability to strip Novozyme 188 immobilized on TMAP and MIEX particles was examined using 1 M NaCl, pH 5.0 (stripping buffer 1) or 1 M NaCl, pH 3.0 (stripping buffer 2). Stripping was performed during 30 min, with shaking at 850 rpm at room temperature and then the residual immobilized beta-glucosidase activity was measured. Subsequently, the stripped particles were subjected to re-binding/re-charging of Novozyme 188 at pH 5.0 according to the standard immobilization conditions described above.

2.5. Immobilization of Celluclast 1.5L on TMAP and MIEX particles

Celluclast 1.5L from Novozymes, is an enzyme mixture containing all the cellulases needed for lignocellulose hydrolysis, and was immobilized on TMAP and MIEX particles. Immobilization was performed as described above using a protein concentration of approximately 1 mg/mL, pH 5.0 for 30 min. The hydrolysis capacity of immobilized cellulases was determined by incubating 9 mg of either TMAP or MIEX particles with immobilized enzymes with 2% (w/v) microcrystalline cellulose (Sigma-Aldrich, St. Louis, MO, USA) in 50 mM acetate buffer, pH 4.8. The hydrolysis was performed at 50ºC for 24 h and the glucose formed was determined with HPLC. In order to study the recyclability of immobilized Celluclast 1.5L, the particles were washed after the first hydrolysis cycle with 20 mM phosphate buffer, pH 7.0 and subsequently used for a second hydrolysis cycle.

2.6. Lignocellulose hydrolysis using free cellulases in combination with immobilized Novozyme 188

In order to study the applicability of immobilized Novozyme 188 on a more complex substrate (compared to PNPG) hydrothermally pretreated (180ºC for 10 min) wheat straw

was utilized. The pretreated wheat straw was kindly provided by Zsófia Kádár from DTU Risø Campus (Roskilde, Denmark) and the composition as reported by them is (% wt/wt DM): Cellulose 48.1, xylan 19.5, arabinan 1.5, lignin 22.1, ash 4.6. During the hydrolysis experiments immobilized Novozyme 188 (using either TMAP or MIEX particles) was combined with free cellulases (Celluclast 1.5L obtained from Novozymes). The experiments were carried out in 2 mL Eppendorf tubes using a water insoluble content (WIS) of 2% (w/v) of pretreated wheat straw suspended in 50 mM acetate buffer (pH 4.8). The mixture contained 0.05% (w/v) NaN_3 to prevent microbial contamination. The enzyme activity loading of Celluclast 1.5L was 10 FPU (filter paper units)/g cellulose and the activity loading of immobilized beta-glucosidase (on either TMAP or MIEX particles) was 20 U/g cellulose. One experiment was also carried out where only Celluclast 1.5L was added (10 FPU/g cellulose) using the same conditions described above. The hydrolysis was performed at 50ºC with gentle mixing using a vertical rotator. After 20 h of incubation the immobilized Novozyme 188 was magnetically separated using a magnetic bar. The supernatant, containing insolubles, was collected, centrifuged (at 14000 x g for 5 min) and the supernatant from this tube was boiled for 10 min to inactivate any residual enzyme. The glucose formed was analyzed with HPLC. After one hydrolysis cycle (20 h), the collected magnetic particles were washed three times with 20 mM phosphate buffer (pH 7.0) and then used for a second hydrolysis cycle using the same conditions as described above with fresh substrate and fresh Celluclast 1.5L (in total four hydrolysis cycles were performed). For each hydrolysis cycle, controls were prepared using the same hydrolysis conditions but excluding addition of enzymes. The glucose yield was determined by equation 1,

$$\frac{[Glucose]}{1.11 \times f \times [Biomass]} \times 100 = Yield\ (\%) \tag{1}$$

where [*Glucose*] is the glucose concentration (g/L) after a hydrolysis cycle, f is the cellulose fraction dry biomass (g/g), [*Biomass*] is the concentration (g/L) of dry biomass at the start of the hydrolysis and 1.11 is the conversion factor of cellulose to glucose.

2.7. Analysis of glucose by HPLC

HPLC was used for analyzing glucose formed from hydrolyzed cellulose. Samples were filtered using a 0.22 µm filter, transferred to HPLC vials and stored at -20ºC until analysis. The column used was a Hi-Plex K column (Agilent Technologies, Santa Clara, CA, USA) operating at 80ºC and the eluent was MilliQ water using a flow rate of 0.7 mL/min. The glucose formed was detected with a 1260 Infinity refractive index detector (Agilent Technologies, Santa Clara, CA, USA) operating at 35ºC.

3. Results and discussion

3.1. Immobilization of Novozyme 188 on anion exchange magnetic particles

In the present work, we have immobilized beta-glucosidase (Novozyme 188 from Novozymes) on two types of anion exchange magnetic particles and examined immobilization conditions, re-charging the particles and the possibility of using the immobilized beta-glucosidase on real lignocellulosic substrate.

The Novozyme 188 preparation originates from *Aspergillus niger* and the isoelectric point of the beta-glucosidase is 4.0 (Watanabe et al., 1992). Hence, using a pH above 4.0 and anion exchange particles should allow adsorption of beta-glucosidase through electrostatic interactions. Different immobilization pH values of pH 5.0, 7.0 and 9.0 were tested on TMAP and MIEX particles and the results are shown in Table 1. It can be observed that beta-glucosidase in Novozyme 188 could be immobilized on both TMAP and MIEX particles and the highest activities were using pH 5.0 (Table 1) and were 67.7 and 30.1 U/g particles,

respectively. It was thought that increasing pH may yield higher binding capacities (mg protein/g particles) and higher bead activities because of the greater ionization of the enzyme. However, the bead related enzyme activity decreased with increasing immobilization pH (Table 1). The specific activity of immobilized Novozyme 188 was also highest using pH 5.0 (3.3 and 1.7 U/mg protein for TMAP and MIEX particles, respectively) but was lower compared to the free enzyme (7.2 U/mg protein). Lower specific activity is common during immobilization and has been reported in several previous studies (Balcão et al., 2001; Gómez et al., 2010; Mohy Eldin et al., 2012). In a previous study we covalently immobilized a purified beta-glucosidase (from Megazyme) on magnetic particles and the specific activity decreased by 69% upon immobilization (Alftrén and Hobley, 2013). The loss in catalytic activity could be due to changes of the enzyme structure upon immobilization, non-favorable micro-environmental conditions and lower accessibility of the substrate to the active site for the immobilized enzyme (due to non-directed immobilization).

Table 1

Immobilized enzyme activity (U/g particles), binding capacity (mg protein/g particles) and specific activity (U/mg protein) of Novozyme 188 immobilized on TMAP and MIEX particles using pH 5.0, 7.0 and 9.0.

Immobilization pH	Immobilized activity (U/g particles)		Binding capacity (mg protein/g particles)		Specific activity (U/mg protein)	
	TMAP	MIEX	TMAP	MIEX	TMAP	MIEX
5.0	67.7	30.1	20.7	17.7	3.3	1.7
7.0	56.3	20.8	29.2	16.5	2.0	1.3
9.0	33.3	12.5	26.4	11.9	1.3	1.1
9.0[a]	11.0[a]	6.9[a]	-	-	-	-

[a] Immobilization of the beta-glucosidase Accellerase BG.

3.2. The effect of binding pH on enzyme recyclability

The recyclability of the enzyme immobilized at pH 5.0, 7.0 and 9.0 was examined with four sequential 5 minute hydrolysis cycles at 50ºC using 5 mM PNPG, pH 4.8. After each cycle, the particles were magnetically separated and supernatant was removed, and the separated magnetic particles were washed twice with 1 mL 20 mM phosphate buffer (pH 7.0) before being recycled. Figure 1A and B shows the normalized activity (using binding pH 5.0, 7.0 and 9.0) as a function of hydrolysis cycles for TMAP and MIEX particles, respectively. It can be observed that the activity does not decrease significantly for both particle types and there is no significant difference if immobilization has been conducted at pH 5.0, 7.0 or 9.0. This confirms that the pH studied during immobilization does not have any significant influence on enzyme leakage and recyclability.

The beta-glucosidase Accellerase BG (from Dupont Industrial Biosciences, Palo Alto, CA, USA) was also examined for immobilization. The beta-glucosidase (gene: bgl1) in this preparation originates from *Trichoderma reesei* and has been reported to have an isoelectric point of 8.5 (Chirico and Brown, 1987). Using the same immobilization conditions as with Novozyme 188 (the binding pH tested was 9.0) the obtained bead enzyme activities for TMAP and MIEX particles were 11.0 and 6.9 U/g particles (Table 1), respectively. The immobilized Accellerase BG was recycled (in the same way as described above) and it was observed that after one recycle the activity had decreased by 79% and 32% for TMAP and MIEX particles, respectively. Given the high pI for Accellerase BG, cation exchange magnetic particles could possibly be more suitable than anion exchangers for immobilization.
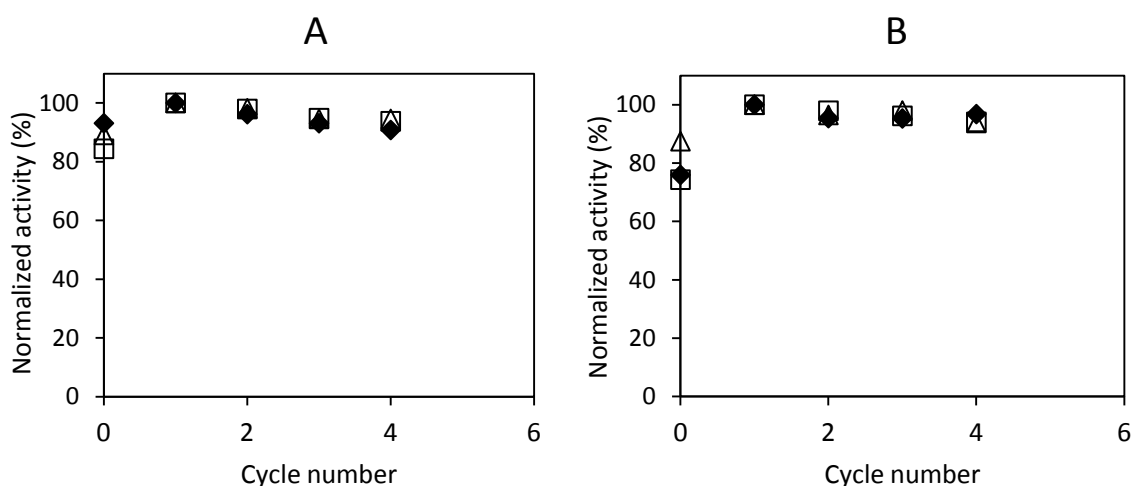

Fig. 1. The effect of immobilization pH on recyclability of Novozyme 188 immobilized on TMAP (A) and MIEX particles (B). Immobilization pH 5.0, filled diamonds; pH 7.0, unfilled squares; pH 9.0, unfilled triangles. One hydrolysis cycle was performed for 5 min, 5 mM PNPG, 50ºC, pH 4.8.

3.3. The effect of adding salt during immobilization

Preliminary experiments showed it was extremely difficult to strip Novozyme 188 from magnetic particles with conventional high ionic strength buffers. Furthermore, the results with Accelerase BG suggest significant non-electrostatic interactions occurred, especially to MIEX beads. Thus, to further examine whether the primary mode of immobilization was by ion exchange, 1 M NaCl was added to the Novozyme 188 preparation prior to immobilization (immobilization conditions: 30 min of binding at pH 5.0, 1 M NaCl, 850 rpm). Under these conditions the binding capacity (in mg protein/g particles) for TMAP and MIEX particles was reduced by 80 and 88%, respectively. This clearly shows that the main interactions were of electrostatic character for both TMAP and MIEX particles and raises the possibility that extreme conditions may allow enzyme stripping. However, there might be other interactions like hydrophobic interaction involved in binding of beta-glucosidase since roughly 20% of enzyme still binds even in the presence of a high concentration of ions.

3.4. Binding capacity as a function of time

The effect of binding time on binding capacity (mg protein/g particles) was studied for both TMAP and MIEX particles, at pH 5.0 and for a total time period of 360 min. Figure 2 shows that already after 5 sec of incubation, quite considerable immobilization of Novozyme 188 on both TMAP and MIEX particles (26.2 and 13.8 mg/g particles, respectively) has occurred. The binding of Novozyme 188 to TMAP particles was more rapid than for MIEX particles and was essentially complete after 60 min. The difference in binding behavior between TMAP and MIEX particles was expected since TMAP are non-porous while MIEX particles are macroporous. Diffusion of the enzyme to the binding sites takes longer for the macroporous MIEX particles and is reflected by a slower immobilization.
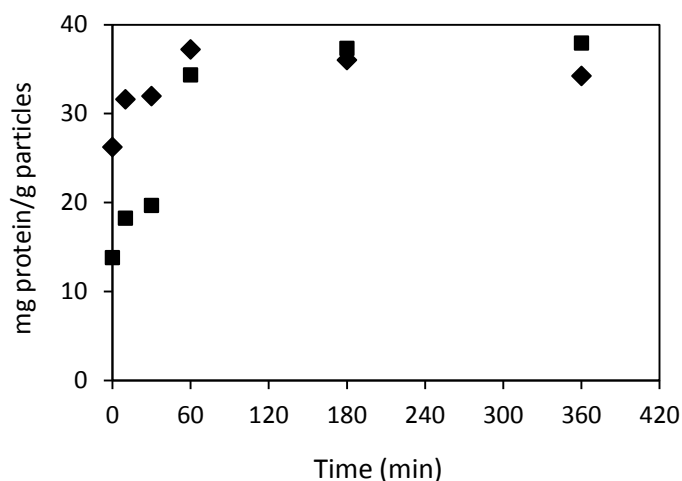
Fig. 2. Binding capacities (mg protein/g particles) versus time (min) for TMAP (filled diamonds) and MIEX (filled squares) particles (binding pH 5.0).

3.5. Stripping and re-binding of Novozyme 188 on TMAP and MIEX particles

In order to further examine the strength of enzyme binding and to investigate the possibility of re-charging the core magnetic particles, Novozyme 188 immobilized on TMAP and MIEX particles was stripped using 1 M NaCl, pH 5.0 (stripping buffer 1) or 1 M NaCl, pH 3.0 (stripping buffer 2). Stripping was performed for 30 min at room temperature with stirring at 850 rpm and the residual immobilized beta-glucosidase activity was measured. For TMAP particles after using stripping buffer 1 and 2, the activity was 31 and 9% of the initial immobilized activity. For MIEX particles using stripping buffer 1 and 2, the activity was 11 and 8% of the initial immobilized activity. Thus, it was possible to strip over 90% of the immobilized beta-glucosidase activity from both TMAP and MIEX particles. Decreasing the stripping pH from 5.0 to 3.0 (i.e. below the isoelectric point) resulted in a higher stripping efficiency. After stripping, the particles were used for re-binding Novozyme 188 employing the standard conditions (pH 5.0, 30 min at room temperature). For both TMAP and MIEX particles it was possible to obtain similar immobilized beta-glucosidase activity (66.5 and 31.8 U/g particles, respectively) as that prior to the stripping step. This demonstrates that the

particles were not impaired and also supports the contention that the enzyme was stripped off to expose binding sites and not just deactivated.

3.6. Immobilization of Celluclast 1.5L on TMAP and MIEX particles

Given the very promising results with Novozyme 188 it was of interest to investigate whether Celluclast 1.5L (Novozymes) could be immobilized on TMAP and MIEX particles in a similar way. Immobilization was performed as described above (for Novozyme 188) using a protein concentration of approximately 1 mg/mL, pH 5.0 and an immobilization time of 30 min. The binding capacity of TMAP and MIEX particles was determined to be 27.9 and 16.7 mg protein/g particles, respectively. Hence, the trend in binding capacity is similar to immobilization of Novozyme 188 with highest capacities obtained using TMAP particles.

The hydrolysis capacity of immobilized Celluclast 1.5L was determined by incubating 9 mg of either the TMAP or MIEX particles (with immobilized enzyme) with 2% (w/v) microcrystalline cellulose in 50 mM acetate buffer, pH 4.8. The hydrolysis was performed at 50ºC for 24 h and the glucose formed was analyzed by HPLC. The hydrolysis yield (glucose yield) was determined to 46 and 37% for TMAP and MIEX particles, respectively. The higher yield of TMAP compared to MIEX particles is consistent with the higher binding capacity of TMAP. In order to study the recyclability of immobilized Celluclast 1.5L the particles were, after the first hydrolysis cycle, washed with 20 mM phosphate buffer (pH 7.0) and subsequently used for a second hydrolysis cycle. After the second hydrolysis cycle the yield had decreased to zero for both TMAP and MIEX particles indicating that the enzymes had either desorbed from the particles or become inactivated. Cellulases (endo- and exocellulases) consist of a cellulose binding domain which mediates the interaction between the enzyme and cellulose (Carrard et al., 2000; Linder et al., 1995). The high affinity between the cellulose binding domain and cellulose is speculated to have promoted desorption of the

cellulases from TMAP and MIEX particles resulting in a glucose yield of zero after the second hydrolysis cycle. Thus an approach using cellulases without cellulose binding domains or using covalent attachment should be investigated.

3.7. Lignocellulose hydrolysis using free cellulase in combination with immobilized Novozyme 188

In order to study the applicability of the immobilized Novozyme 188 on a more complex substrate, pretreated wheat straw was utilized (the composition is reported in Materials and methods). Immobilized Novozyme 188 (on either TMAP or MIEX particles) was combined with free cellulases (Celluclast 1.5L) using a WIS content of 2% (w/v) and incubation time of 20 h at 50ºC, pH 4.8. Figure 3 shows that the hydrolysis yield using only Celluclast 1.5L is 46%, while using Celluclast 1.5L with added immobilized Novozyme 188 on TMAP and MIEX particles is 64% and 67%, respectively. The results indicate that immobilized Novozyme 188 can be used to boost cellulose hydrolysis on more complex substrates (compared to PNPG) such as pretreated wheat straw. After 20 h of enzymatic hydrolysis, the immobilized Novozyme 188 was magnetically separated, washed with 20 mM phosphate buffer (pH 7.0) and subsequently used for a new hydrolysis cycle with fresh substrate and fresh Celluclast 1.5L. In Figure 3 it can be observed that immobilized Novozyme 188 on MIEX particles could be used to increase the hydrolysis rate of free cellulases for at least four hydrolysis cycles. However, for Novozyme 188 immobilized on the small 100 nm TMAP particles recycling was not possible because of interactions with residual insolubles consisting of lignin, hemicellulose and non-hydrolyzed cellulose which inhibited separation of the magnetic particles. For the large ~200 µm MIEX particles no such interaction was observed and thus the magnetic separation of the immobilized Novozyme 188 was successful and after four hydrolysis cycles the loss of particles was measured and found to be less than

5% (compared to the initial amount). The beta-glucosidase activity was measured subsequent to the fourth hydrolysis cycle and it was determined to be 17.7 U/g particles (using the PNPG assay). Thus, the immobilized beta-glucosidase activity had decreased by 41% (initial activity was 30.1 U/g particles). By re-binding Novozyme 188 (binding pH 5.0, mixing at 850 rpm for 30 min) on the used MIEX particles (after the fourth cycle) a bead activity of 28.7 U/g particles was obtained, which is very close to the initial value. The possibility of re-charging base particles is economically beneficial since it can save costs associated with particle production in a way that is not possible with covalently immobilized enzymes. In addition to the possibility of re-charging base particles, the MIEX anion exchange particles are extremely cheap. In a previous study we covalently immobilized Novozyme 188 on cyanuric chloride activated magnetic particles from Chemicell (and obtained a bead activity of 9.8 U/g particles) (Alftrén and Hobley, 2013). The base particles used in that work are produced for small-scale lab applications and the costs for these (with 80% discount) are approximately 110 €/g particles yielding a cost of ca. 11.2 €/Unit of immobilized beta-glucosidase activity (considering materials costs only). On the contrary, for MIEX particles the cost for immobilized beta-glucosidase activity is estimated to be approximately 3-4 orders of magnitude lower (for TMAP the cost is about 2 orders of magnitude lower), making them much more suitable for use in large scale applications such as in the production of lignocellulosic derived biochemicals. Each recycle reduces the costs further. Whilst the maximum number of recycles shown here is 4 times, we have demonstrated that TMAP and MIEX can be re-used 50 times for protein purification (unpublished results).
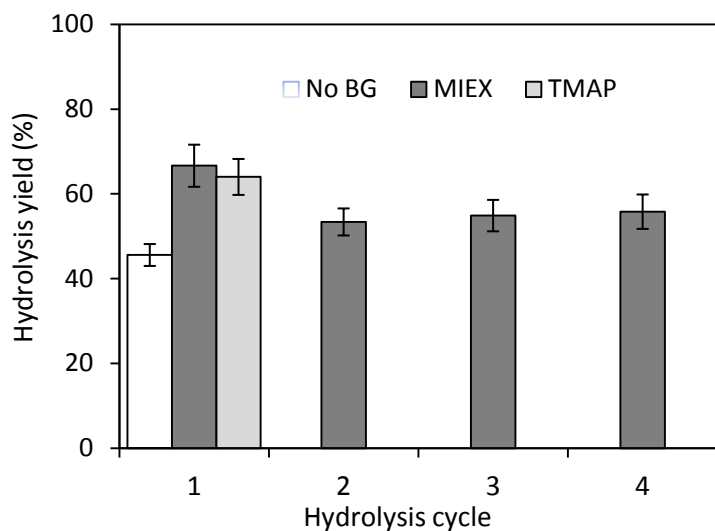
Fig. 3. Recycle study using immobilized Novozyme 188 on TMAP and MIEX particles in combination with free Celluclast 1.5L. One hydrolysis cycle was performed for 20 h using a WIS content of 2% (w/v) pretreated wheat straw, pH 4.8 (50 mM acetate buffer) at 50ºC. Enzyme activity loading of Celluclast 1.5L and immobilized beta-glucosidase was 10 FPU/g cellulose and 20 U/g cellulose, respectively. No BG (beta-glucosidase) = Celluclast 1.5L only. Fresh Celluclast 1.5L and wheat straw were added to each new hydrolysis cycle. Data and error bars represent average and standard deviation, respectively, of 3 replicate experiments.

4. Conclusions

Novozyme 188 can be immobilized by simple adsorption on anion exchange magnetic particles and used for repeated hydrolysis of pretreated wheat straw. The immobilized activity (U/g particles) and specific activity (U/mg protein) are highest at an immobilization pH of 5.0 for both TMAP and MIEX particles. It is possible to strip the immobilized enzyme and rebind with fresh Novozyme 188; demonstrating the potential for reducing costs of particle manufacturing by re-charging base particles. Adding magnetic immobilized beta-

glucosidase to free cellulases boosts the hydrolysis yield of pretreated wheat straw and it is possible to re-use the enzyme, and retain activity, for at least four consecutive hydrolysis cycles. Future work should focus on scale-up experiments and techno-economic analysis of the application of magnetically recyclable beta-glucosidase.

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Paper V

Technical Report

Pilot scale immobilization of beta-glucosidase to magnetic particles and demonstration of multi cycle re-use

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Abstract

A study of immobilization of beta-glucosidase (Novozyme 188 from Novozymes) on magnetic ion exchange (MIEX$^®$) particles and repeated use of the immobilized enzyme in pilot scale is described. A prototype Magnetically Enhanced Centrifuge (MEC) was used for the magnetic separations. In an integrated process, beta-glucosidase (Novozyme 188 from Novozymes) was immobilized by simple adsorption to a large amount (400 g) of low-cost MIEX particles originally designed for treatment of drinking water. The immobilized enzyme was incubated at 50°C with 2% w/v cellulose for 20 h together with free Celluclast 1.5L. The immobilized enzyme was then efficiently separated from the cellulose by the MEC. Beta-glucosidase immobilized on MIEX particles initially had an activity of 60.3 U/g particles and 33% of the immobilized enzyme activity was lost after the first cycle of heating and mixing with cellulose. During the following two incubation cycles only a minor further loss (~5%) of beta-glucosidase activity was observed. After three cycles of magnetic separation there had only been a loss of 7.8% of the MIEX particles themselves.

Keywords: Enzyme immobilization, High Gradient Magnetic Separation (HGMS), magnetically enhanced centrifugation (MEC)

1. Introduction

There is great interest in exploring ways to re-use enzymes in industrial processes for better sustainability, for example in the production of lignocellulosic based bioethanol [1]. Enzymes constitute a major expense for converting biomass to ethanol [2] and more efficient methods of using enzymes are needed to reduce costs. A promising recent technology to allow re-use of enzymes is immobilization on magnetically susceptible particles [3-5]. Magnetic particles can be selectively separated from solutions containing other suspended solids by application of a magnetic field [6]. This feature facilitates repeated use of magnetic immobilized enzymes in lignocellulosic material containing insolubles.

Beta-glucosidase is an important enzyme in the hydrolysis of lignocellulosic biomass and this enzyme has recently been immobilized to magnetic particles by covalent or affinity interactions and successfully re-used in ca. 1 mL volume lab-scale studies [5,7]. Magnetic particles are easy to work with in bench scale where a simple bar magnet can be applied for separation. However, this approach cannot be applied directly at large scale and high gradient magnetic separation (HGMS) is needed. A HGMS system consists typically of a wire matrix which distorts an externally applied magnetic field creating high magnetic field gradients, which aggregate and attract magnetic particles fed into the matrix [8]. HGMS has been applied in various proof-of-principle bioseparations with functionalized magnetic particles to e.g. selectively capture proteins from crude feedstocks [9-11], separate cells [12], remove environmental contaminants such as radioactive nuclides in transuranic waste [13] or remove soluble organic compounds from wastewater [14]. However, these are almost exclusively batch wise separations and continuous operation is needed for processing large volumes. A further limitation of many HGMS system designs for bioprocessing is that the magnetic filter matrices can become blocked by non-magnetic insolubles [15]. A magnetic centrifuge (MEC) has recently been reported that can potentially solve the limitations of previous HGMS

3

systems [16-18]. The MEC is designed as a centrifuge placed inside a strong electromagnet. In the middle of the centrifuge bowl is a matrix of stacked magnetic wires analogous to the stacked discs in a disc-stack centrifuge, which can also rotate. With the electromagnet switched on, the magnetic wire array creates high magnetic field gradients which attract and capture magnetic particles. However, due to their rotation the cake of magnetic particles on each wire is slung off and accumulates on the sides of the bowl. In this way the wire arrays never become saturated with magnetic particles. If bowl rotation and thus g-forces at the wall are low enough, non-magnetic materials pass straight through and the magnetic materials remain in the centrifuge. When the magnetic field is switched off the magnetic particles are released and can be recovered [18]. Although not available on MECs hitherto described, continuous discharge of magnetic particles by bowl separation or nozzles could potentially be used for truly continuous separation.

The objective of this study was to demonstrate for the first time at pilot scale the potential of magnetic immobilized enzymes for biofuels and biorefineries applications. To be of industrial relevance an extremely cheap immobilized enzyme is needed and thus commercial beta-glucosidase (Novozyme 188 from Novozymes) and the cheap anion exchange magnetic particles (MIEX®) from Orica Watercare [19-21] were used for its construction.

2 Materials and methods

2.1 Magnetic enhanced centrifuge

The MEC used here was similar to that described by Lindner et al. (2013) but scaled-up from a matrix diameter of 100 mm and centrifuge volume of 1.25 l to a matrix diameter of 198 mm and bowl volume of 6 L by Andritz KMPT GmbH (Vierkirchen, Germany). The separator consisted of a centrifuge bowl with a stacked array of magnetisable wires radiating out from a hollow central axle and an external electro magnet of 0.4 T. A schematic of the setup is

shown in figure 1. The centrifuge bowl was 200 mm in diameter and was not magnetisable and was capable of rotating at 3500 RPM (i.e. ca.1370 g) in a clockwise direction, but in all the work done here was held stationary. The wire array was capable of rotation at speeds independent of the bowl in a clockwise direction at a maximum speed differential with respect to the bowl of 120 rpm i.e. allowing 120 rpm with a stationary bowl. Liquid flow into the bowl of the MEC was from below, displacing liquid or air present, and overflowed out down through the top of the central hollow axle and out to waste or into a recirculation loop (total volume of system with empty feedtank was 12 L).

2.2 Immobilization of beta-glucosidase on MIEX particles

The magnetic ion exchange resins MIEX from Orica Watercare are macroporous with a diameter of 150-180 μm [22] and a density of 1.05 g/cm3. Prior to immobilization of beta-glucosidase, 400 g of MIEX particles were equilibrated for 10 min with 0.5 M sodium sulphate (~20 g particles/L) with stirring followed by four repeated washing steps with deionised water (~20 g particles/L) for 10 min to remove excess ions. MIEX particles settled rapidly due to their size and density and this feature was exploited for isolation of the particles, allowing the supernatant to be removed between the washing steps. Enzyme immobilization was performed in a 50 L tank by mixing MIEX particles with beta-glucosidase (Novozyme 188; Novozymes, Bagsværd, Danmark) at a concentration of 0.05 g beta-glucosidase per g particles (total volume of 20 L) for 60 min with stirring (410 rpm) at room temperature. The pH during binding was adjusted to 5.0 with 2 M NaOH. After 60 min of binding, the immobilization was terminated by magnetic capture of the particles by a Magnetic Enhanced Centrifuge (MEC).

2.3 Magnetic separation of immobilized beta-glucosidase

The suspension of MIEX particles after beta-glucosidase binding was pumped into the MEC with a flow rate of 240 L/h. The magnetic particles were captured and separated from the solution containing unbound enzyme with the magnetic field turned on. The unbound enzyme flowed through the MEC into a waste stream. 20 L of deionised water was then added to the feed tank of the MEC and pumped through the separator at 240 L/h with the magnet turned off and allowed to recirculate (Figure 1). During this procedure the array of wires rotated at 120 rpm to aid release of the magnetic particles. After 10 min of washing and recirculation through the MEC and 50 L tank (Figure 1), the electromagnet was turned on and the matrix rotation turned off whilst recirculating the suspension to capture the immobilized enzyme. When no immobilized enzyme could be observed in the outlet flow, the supernatant was pumped to waste. After the first wash, the wash procedure was repeated using 20 L of 25 mM acetate buffer, pH 4.8. After this final wash the immobilized enzyme was trapped in the MEC with the magnet turned on and matrix rotation turned off. The supernatant was tested for residual enzyme activity by the PNPG assay and after that sent to waste. The immobilized enzyme was then released from the MEC by turning off the electromagnet, setting the matrix to 120 rpm and washing through the MEC with 80 L of 25 mM acetate buffer, pH 4.8. The particles were collected in a container and the supernatant was removed, leaving approximately 10 L of 25 mM acetate buffer with 400 g immobilized enzyme.
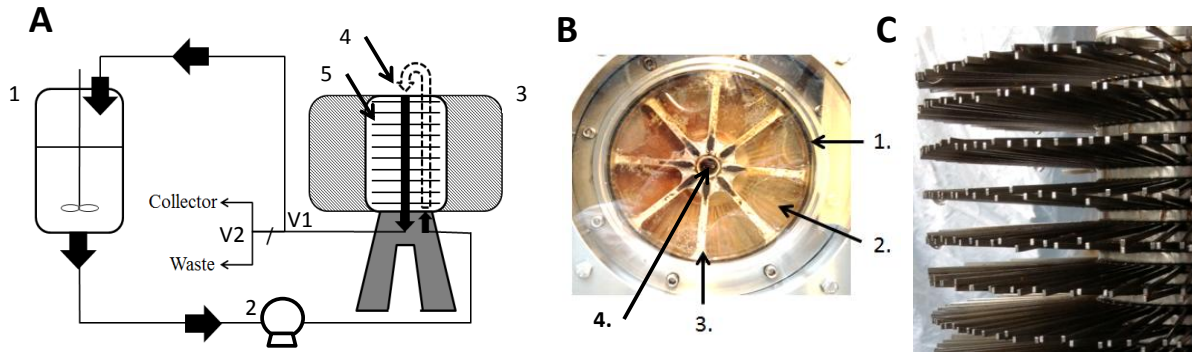
Figure 1. Magnetic centrifuge (MEC) and process setup. A. Illustration of the setup with: 1, feed tank with overhead mixer; 2, mono pump in recirculation loop with valves V1 and V2; 3, electromagnet of MEC; 4, central pipe for suspension exit from MEC; 5, wire matrix in MEC. B. A picture taken through the glass top of the MEC during operation showing: 1, G glass lid; 2, rotating wire matrix; 3, glass lid support; 4, central pipe for suspension exit. C. Uncleaned wire matrix removed from MEC directly after final wash and release of immobilized enzyme in 3rd hydrolysis cycle.

2.4 Repeated hydrolysis cycles using immobilised beta-glucosidase

The separation of immobilized beta-glucosidase from cellulose was studied by using 24 L of a suspension of 2% (w/v) microcrystalline cellulose (Sigma-Aldrich, 435236) in 25 mM acetate buffer, pH 4.8 with 0.025% (w/v) ProClin (Sigma-Aldrich, 49376-U) to inhibit any microbial growth. To this was added free cellulases (Celluclast 1.5L obtained from Novozymes) with an activity of 10 FPU (filter paper units) per gram cellulose and the magnetic immobilized beta-glucosidase (60 U/g cellulose). The suspension was incubated at 50°C with stirring from an industrial overhead mixer (410 rpm) and after 20 h samples were taken out for analysis of residual immobilized enzyme activity. The immobilized enzyme was separated from the cellulose particles using the MEC. This was done by pumping the suspension from the 50 L feed tank at 240 L/h into the MEC with the electromagnet turned

on and matrix rotation turned off thus trapping the magnetic immobilized enzyme. The supernatant was recycled until all the magnetic immobilized enzyme was trapped by the separator and the clear supernatant pumped to waste (ca. 10 min). Acetate buffer (40 L, 5 mM, pH 4.8) was added to the feed tank and pumped into the MEC at 390 L/h with the field on and the matrix at the lowest speed (20 rpm) to partially suspend the magnetic immobilized enzyme and aid removal of entrained cellulose particles. No immobilized enzyme was lost during this procedure and the clear supernatant was sent to waste (ca. 10 min). Acetate buffer (20 L of 5 mM, pH 4.8) was then added and re-circulated between the feed tank and the MEC for 1 min with the magnet turned off and the matrix at maximum speed of 120 rpm to re-suspend trapped cellulose and immobilized enzyme. The magnet was then turned on to trap the immobilized enzyme in the MEC. This wash procedure was repeated 5 times until the supernatant was clear of cellulose particles as determined by a visual inspection. The final wash of the immobilized enzyme was with 20 L of 25 mM acetate buffer, pH 4.8 and they were displaced from the MEC as described earlier. The immobilized beta-glucosidase was then used for a new hydrolysis without further treatment. Three consecutive hydrolysis cycles were performed using the same conditions and reusing the immobilized beta-glucosidase.

2.5 Assay of immobilized beta-glucosidase activity

The activity of immobilized beta-glucosidase (U/g particle) was assayed using *p*-nitrophenyl-β-D-glucopyranoside, PNPG (Sigma-Aldrich, St. Louis, MO, USA), based on a previously described method for free beta-glucosidase [23] as described by us earlier [5]. In brief, 5 mM PNPG in 50 mM sodium acetate buffer (pH 4.8) was mixed with the immobilized beta-glucosidase sample (0.5-1.0 mg particles) and incubated at 50ºC for 5 min with mixing. The immobilized enzyme was then magnetically separated and 1 M Na_2CO_3 was immediately added to the supernatant to terminate any remaining reaction. The liberated *p*-nitrophenol

(PNP) was measured at 405 nm. One unit of beta-glucosidase activity (U) releases 1 μmol of PNP per min under the assay conditions.

3 Results and discussion

3.1 Immobilization of beta-glucosidase on MIEX particles and reusing the immobilized enzyme at pilot scale.

There are previous studies of immobilization of enzymes to magnetic particles by adsorption [24, 25]; however, none have demonstrated the potential at pilot scale. The beta-glucosidase (Novozyme 188 from Novozymes) used for immobilization in this study is a homodimer of 240 kDa from *Aspergillus niger* with an isoelectric point of 4.0 [26]. We have earlier shown in laboratory studies comparing the MIEX particles with TMAP particles from Merck that extremely strong adsorption of beta-glucosidase can be achieved at pH 5.0, primarily due to electrostatic interactions (manuscript submitted). In the current study we have therefore used the same pH and have obtained approximately twice as high enzyme activity immediately after immobilization (60.3 U/g MIEX particles) as compared to our earlier study. This was most likely due to the longer time for adsorption (60 instead of 30 min) and better mixing, both of which are important when using large porous particles such as the MIEX type, where diffusion of the enzyme to the binding sites in the pores must occur. To examine the ability to reuse the immobilized enzyme, a simulated lignocellulose hydrolysis was conducted in which the immobilized enzyme was heated to 50°C and mixed with cellulose particles with a mean size of 20 μm, which is approximately 8 times smaller than the MIEX particles (150-180 μm). A mix of cellulases (Celluclast 1.5 from Novozymes) was added, leading to partial hydrolysis of the cellulose particles during the 20 h incubation. Approximately 33% of the beta-glucosidase activity was lost after the first cycle, but thereafter the activity remained

almost constant for the following three cycles with a minor further loss of 5% from the first to the third cycle (Figure 2). Subsequent to the last cycle the immobilized beta-glucosidase was stored at 4ºC (acetate buffer, pH 4.8) and it was observed that the activity was not reduced after 3 months of storage (Figure 2). The results demonstrate that binding of beta-glucosidase to the MIEX particles was very strong considering that the immobilization was done by simple adsorption to an anion exchanger. It is speculated that the first initial 33% drop in activity was due to desorption of loosely bound enzyme. However, other effects such as fouling with cellulose particles were also seen, which would artefactually increase the apparent weight of each immobilized enzyme particle, thus reducing the apparent specific activity measured on a units per gram of bead basis.

The mass of magnetic immobilized enzyme was determined before the first and after the third cycle by taking out a sample of a known volume, washing once with deionised water and then drying the particles at 100°C to constant weight. It was found that after 3 cycles the mass of immobilized enzyme had been reduced from 400 g to 369 g, i.e. a loss of 7.8%. Given that each cycle involves, in total, 6 separations (1 recovery and 5 washes), and that 18 separations had been carried out with no dismantling or manual cleaning of the MEC, the separation (and subsequent release) efficiency was 99.55% (i.e. 0.45% of particles were lost per separation cycle). Reducing the number of washing steps, careful optimization of the balance between flow rate during capture and magnetic array rotation for release of entrained non-magnetic particles, combined with a small strong 'police' HGMS filter in the waste stream could potentially be used to decrease the overall immobilized enzyme loss over many hydrolysis cycles to insignificant levels.
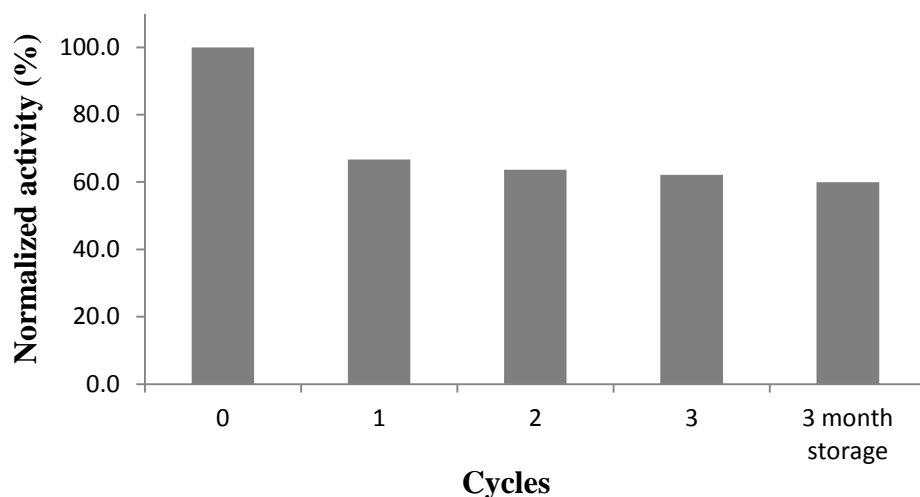
Figure 2. Activity of magnetic immobilized beta-glucosidase during three consecutive cycles of hydrolysis with 2% w/v cellulose at 50°C for 20 h. The activity of immobilized beta-glucosidase has been normalized to the initial activity of beta-glucosidase (i.e. 60.3 U/g particles). The activity of immobilized beta-glucosidase was 40.2, 38.4 and 37.5 U/g particles in cycle 1, 2 and 3, respectively. The column '3 month storage' shows the residual immobilized activity after 3 months (at 4ºC, acetate buffer, pH 4.8) of storage subsequent to the 3rd cycle. After each cycle the amount of glucose released was measured and found to be 9.5 g/L, 9g/L and 10.5 g/L respectively.

3.2 Suitability of the magnetically enhanced centrifuge for separation of magnetic immobilized enzyme

The theory behind the MEC and characterization of magnetic adsorbent separation performance in buffers for capturing an enzyme in a single protein system using a laboratory prototype of the device used here was recently described by Lindner et al [17,18]. However in the present work, a larger modified device without peeler knives and which is ready for commercialization has been used (Figure 1) for separating magnetic immobilized enzyme

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from non-magnetic cellulose particles. It is thus important to report our observations of its performance in this more realistic system. The process setup employing the MEC used in the current study is very simple as can be seen in Figure 1A. The MEC was found to easily separate the MIEX based magnetic immobilized enzyme after binding of the beta glucosidase from the non-solids containing buffer solution. When challenged with the cellulose particle suspension, capture of the magnetic immobilized enzyme was also rapidly and efficiently accomplished using a stationary bowl and matrix. However, washing of the immobilized enzyme was more difficult. In a real situation such as a lignocellulose hydrolysis, carryover to the next batch of particles not representing substrate (e.g. lignin) may not be desired. It was found that careful use of matrix rotation (Figure 1A, B) to create turbulence during capture of the magnetic particles in the washing steps was necessary. The magnetic field retained the magnetic enzyme but the turbulence and particle fluidization of the upward liquid flow allowed elutriation of the non-magnetic particles of cellulose. Nevertheless, five wash cycles with 20 L 25 mM acetate buffer were needed to separate all of the cellulose from the immobilized enzyme. Moreover, the mixing effect of the matrix was extremely efficient (> 99% as stated above) for the release of magnetic particles when re-circulating with the field off for wash and displacement, in stark contrast to most HGMS systems (see e.g. [9,15,27]. When the MEC was dismantled for inspection after the final cycle, no magnetic particles were observed in the bowl or attached to the wires (Figure 1C). Efficient separation and release of magnetic particles in large scale in the presence of non-magnetic solids present is not trivial and the MEC appears to overcome the fouling problems, magnetic particle release problems and potentially the capacity problems of magnetic filters reported earlier [28]. In contrast to the work of Lindner et al. [17], the centrifuge bowl was not rotated in the current work and only the wire matrix was used for capture of the magnetic particles. However, the g forces caused by bowl rotation can be used to increase the magnetic particle holding capacity

and if this was combined with bowl separation or nozzles in a similar way to a disk stack centrifuge, the MEC appears to offer the prospect of truly continuous operation, in contrast to the semi-continuous modes that are only possible with all other magnetic separators [6,28].

4 Concluding remarks

Beta-glucosidase can be immobilized to MIEX anion exchange adsorbents giving a robust immobilized enzyme. The immobilized beta-glucosidase can be re-used for at least three consecutive cycles of 20 h each during hydrolysis of cellulose at 50°C. A magnetically enhanced centrifuge is the separator of choice for high efficiency, high throughput magnetic immobilized enzyme separation from solids containing substrates at pilot scale. Further trials at pilot and larger scale are now warranted, however development of a continuous magnetic solids discharge MEC is needed for full scale trials.

Acknowledgements

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Appendix 1.

Recombinant production of a biotin-6His-tag beta-glucosidase in *E. coli* – Optimization of protein expression and purification

**Appendix 1.**

Recombinant production of a biotin-6His-tag beta-glucosidase in *E. coli* – Optimization of protein expression and purification

In paper II a beta-glucosidase from *Bacillus licheniformis* was expressed and *in vivo* biotinylated in *E. coli* and subsequently immobilized directly from cell lysate on streptavidin coated magnetic particles. *In vivo* biotinylation was mediated by fusing the Biotin Acceptor Peptide to the C-terminal of beta-glucosidase and co-expressing the BirA biotin ligase. The approach enabled simultaneous purification and immobilization of the enzyme from crude cell lysate on magnetic particles because of the high affinity and strong interaction between biotin and streptavidin. In the paper different optimization experiments were performed and the results of these are presented in the present appendix section.

Cultivation conditions

An inoculum of *E. coli* BL21(DE3) cells harboring plasmid pACYC184 and pTwin1-BglH-BAP was prepared and incubated overnight at 37ºC, in LB medium (pH 7.0) supplemented with 100 µg/ml ampicillin and 10 µg/ml chloramphenicol, and 10 mM $MgCl_2$. A 5 ml aliquot of the resulting preculture was subsequently used to inoculate 100 ml LB medium supplemented as previously described, and the resulting mixture was incubated at 37ºC under shaking. When OD_{600} reached 0.7, biotin was added to a final concentration of 50 µM, while IPTG was added to induce expression at a final concentration between 0.05-1.0 mM. The beta-glucosidase activity (U/mg total protein) was optimized by varying the IPTG concentration (Figure A1) and induction temperature (Figure A2). It can be observed that the optimal IPTG concentration and induction temperature was 0.2 mM and 22ºC, respectively. These optimal cultivation conditions were thus used during the 3-L batch fermentation described in paper II.
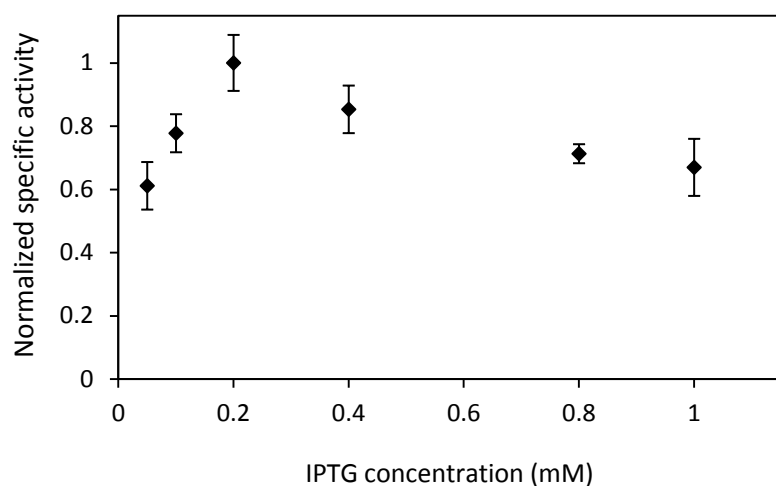
Figure A1. Normalized specific activity as a function of IPTG concentration (mM) during induction. Data and error bars represent average and standard deviation of 3 replicate experiments.
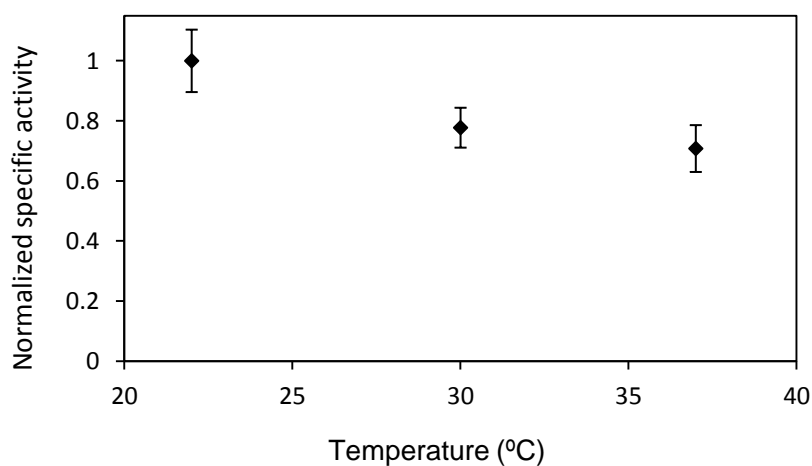

Figure A2. Normalized specific activity as a function of temperature during induction. Data and error bars represent average and standard deviation of 3 replicate experiments.

Immobilized metal affinity chromatography (IMAC) of free beta-glucosidase

Before characterization of the free biotinylated beta-glucosidase, the enzyme was purified using a HiTrap IMAC FF 1ml column (GE Healthcare, Uppsala, Sweden) charged with Ni^{2+}. The flow was delivered by a syringe pump (Harvard Syringe pump Type 22, Harvard Apparatus, Holliston, MA,

USA) set at 1 ml/min and the flow through was collected using a fraction collector (Helirac 2212, LKB, Bromma, Sweden) set at 0.5 ml/tube. Prior to use the column was equilibrated with 5 column volumes (CV) of equilibration buffer (20 mM sodium phosphate, 0.5 M NaCl, pH7.4) and subsequently loaded with 10 CV of sample. Then the column was washed with 10 CV of washing buffer. Two different washing buffers were assessed; washing buffer 1 consisted of 20 mM sodium phosphate, 0.5 M NaCl (pH 7.4), and washing buffer 2 consisted of 20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole (pH 7.4). The bound enzyme was eluted with 5 CV of elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH7.4). The collected fractions were subsequently analyzed for protein content and enzymatic activity as described in paper II. Figure A3 shows an SDS-PAGE of cell lysate, eluate from IMAC column using washing buffer 1 and eluate from IMAC column using washing buffer 2. It can be observed that the purity of beta-glucosidase is increased when using washing buffer 2. As can be seen in Table A1 the specific activity was highest when using washing buffer 2, confirming the results in Figure A3. The enhanced purification obtained when using washing buffer 2 is due to removal of loosely bound proteins when including 10 mM imidazole.
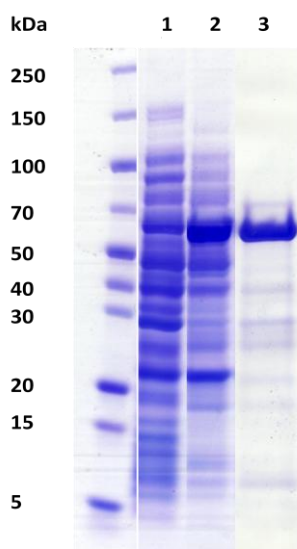
Figure A3. SDS-PAGE showing the purification of beta-glucosidase using IMAC. Lane 1, induced *E. coli* BL21(DE3) cells harboring plasmid pACYC184 and pTwin1-BglH-BAP. Lane 2, eluate from the IMAC column using washing buffer 1 (20 mM sodium phosphate, 0.5 M NaCl, pH7.4); lane 3, eluate from the IMAC column using washing buffer 2 (20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, pH7.4).

Table A1. Specific activity and purification fold for IMAC purified beta-glucosidase. Enzyme activity was determined using the PNPG assay.

Step	Specific activity (U/mg protein)	Purification (fold)
Cell lysate	0.017	na
IMAC purification (washing buffer 1)	0.071	4.17
IMAC purification (washing buffer 2)	0.095	5.59

na. Not applicable.