β-GLUCOSIDASE IMMOBILIZATION ON SYNTHETIC SUPERPARAMAGNETIC MAGNETITE NANOPARTICLES AND THEIR APPLICATION IN SACCHARIFICATION OF WHEAT STRAW AND *Eucalyptus globulus* PULPS.

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ABSTRACT

β-glucosidase from *Trichoderma reesei* was immobilized on synthetic superparamagnetic magnetite (Fe_3O_4) nanoparticles with a mean diameter of 10 nm and were used to supplement cellulase in the enzymatic hydrolysis of three substrates: wheat straw pretreated by steam explosion, *Eucalyptus globulus* pretreated by hydrothermolysis and pulp from hydrothermolysis followed by an alkaline extraction. The hydrolysis yields for each pretreated material, using immobilized β-glucosidase and free cellulase, were 76.1%, 83.6% and 75.6%, respectively and resulted in an improved hydrolysis yields compared with only cellulase. These yields were at most 10% lower than yields reached with free enzymes. The immobilized β-glucosidase was magnetically recovered and reused twice. The differences in the hydrolysis yields were not significant ($p>0.05$) in the case of steam exploded wheat straw and pulp from hydrothermolysis and alkaline extraction. The immobilization of enzymes provides an opportunity to reduce the costs of enzymes in the bioethanol production process.

Keywords: magnetite. β-glucosidase nanoparticles, superparamagnetism, immobilization, saccharification.

1. INTRODUCTION

Lignocellulose is the most abundant renewable biomass produced by photosynthesis and its bioconversion to ethanol requires three basic process steps: a pretreatment process to reduce substrate recalcitrance; enzyme catalyzed hydrolysis of cellulose and hemicellulose components to fermentable sugars; and fermentation to produce ethanol [1].

Enzymatic hydrolysis of cellulose is the process catalyzed by a group of enzymes denominated cellulases composed by endoglucanase, exoglucanases and β-glucosidase [2]. Cellulases are produced extracellulary by a large number of microorganisms including fungi, actinomycetes and bacteria [3]. Cellulase from *Trichoderma reesei* is the most common choice for enzymatic hydrolysis in bioethanol production due to its high level of extracellular cellulase [2, 4], however the main limitation of *T. reesei* cellulase is the relatively low amount of the β-glucosidase, enzyme required to reduce the accumulation of cellobiose which inhibits the endo and exoglunanases enzymes.

Since covalent bonds provide the strongest linkages between enzyme and carrier [5], covalent enzymatic immobilization presents an opportunity to retain and recycle de enzymes, thus reducing the costs of catalyst. Since covalent bonds provide the strongest linkages between enzyme and carrier [5], covalent enzymatic immobilization presents an opportunity to retain and recycle enzymes, reducing the costs of catalyst. Functionalized magnetite (Fe_3O_4) micro and nanoparticles have been widely applied to immobilize several enzymes [6-9], including cellulase [10-13]. The magnetic property of the bioconjugates enzyme/magnetite nanoparticles should improve the recovery of the enzyme magnetic support system in biotechnology applications, such as the saccharification process of pretreated lignocellulosic materials used in bioethanol production.

In the present work, we studied the saccharification process catalyzed by a commercial cellulase complex supplemented by covalent immobilized β-glucosidase enzyme on synthetic superparamagnetic magnetite nanoparticles.

2. MATERIALS AND METHODS

2.1 Covalent Immobilization of β-glucosidase

Superparamagnetic magnetite nanoparticles with a mean diameter of 10 nm were synthesized according to Valenzuela et al. (2010) [14].

Covalent bonding between enzymes and carriers is based on a chemical reaction between the active amino acid residues (AAR) on the enzyme surface and the active functional groups that are attached to the carrier's surface [5]. The first step to immobilize the enzyme was to amino-functionalize the nanoparticles. Thus, 1.5 g of dry magnetite was re-dispersed in 200 mL of ethanol for 30 min by sonication. Then, 2.0 mL of 3-aminopropyltriethoxysilane (3-APTS) were added and this solution was kept at room temperature under magnetic stirring overnight. Silanized nanoparticles were magnetically harvested and washed repeatedly with ethanol and dried under vacuum at room temperature. Fourier transform infrared spectroscopy (FT-IR, Perkin Elmer) was used to confirm chemical bond formation between active Fe_3O_4 and 3-APTS. The magnetic properties of magnetite uncoated and coated by 3-APTS were analyzed at 300 K using a MPMS superconducting quantum interference device magnetometer (SQUID, Quantum Design, XL model, applied field ± 20 kOe). One g of dry amino-functionalized magnetite was re-dispersed by sonication in 100 mL of 5% v/v glutaraldehyde 50 mM phosphate buffer (pH = 7.4) and later stirred overnight at room temperature. The nanoparticles were then magnetically harvested and washed with buffer solution. Afterwards the nanoparticles were combined with a 50 mL solution of 2% v/v of β-glucosidase (Novozymes NS50010) in buffer solution. The mixture was gently stirred overnight at room temperature. Immobilized β-glucosidase on magnetite nanoparticles were magnetically separated and washed and re-suspended in buffer solution and stored at 4ºC until used. The supernatant and the washing solutions were used to determine the unbound proteins concentration. Immobilized proteins concentration was calculated from the difference between protein concentration in the initial solution and the unbound proteins concentration in the supernatant and washing solutions. These concentrations were determined using the Bio-Rad Protein assay kit based on the Bradford's method using bovine serum albumin (BSA) as protein standard.

β-glucosidase specific activity was determined by hydrolysis of p-nitrophenyl β-D-glucopyranoside and expressed as International Units per mg of protein (IU mg^{-1}), [15,16]. Cellulase (Novozymes NS50013) activity was determined by the IUPAC procedure [17].

The percentage of hydrolysis of cellobiose (2.5 g L^{-1}) in 50 mM sodium citrate buffer (pH = 4.8) was used to determine the stability of immobilized enzyme for 45 days.

2.2 Raw Material and Pretreatments

Wheat straw was chipped and pretreated by steam explosion (WS-SE) at 205º C for 5 min.

Eucalyptus globulus chips were pretreated by hydrothermolysis (EG-H) at 180 ºC for 15 min in 1 gal reactor (Parr Instruments, Moline IL, USA). Part of the pulp was delignified by alkaline extraction (EG-HA) by mixing it with 8% NaOH solution at 68 ºC for 1 h.

Chemical characterization of raw and preteated materials was performed following TAPPI Standard Method [18] and Puls et al. (1985) [19].

2.3 Enzymatic Hydrolysis

Enzymatic hydrolysis were performed using a commercial preparation of *T. reesei* cellulase complex (61.0 FPU mL^{-1}). Twenty FPU of cellulase per g of dry pretreated material was used, and was supplemented with immobilized β-glucosidase (I-βG) on 1.0 g of silanized magnetite. Control experiments were carried out using 20 FPU of cellulase per g of dry pretreated material supplemented with free β-glucosidase (F-βG) at the same protein concentration that I-βG, and without supplementation of β-glucosidase.

Enzymatic hydrolysis of the pretreated materials at 5.0% (w/w) of consistency in 50 mM sodium citrate buffer (pH = 4.8) were performed in shaker at 160 rpm and 50º C for 72 h. Samples were taken at 6, 12, 24, 48 and 72 h. The content of cellobiose and the glucose released by the enzymatic hydrolysis were analyzed by HPLC [19]. The hydrolysis yield was expressed as the percentage of glucose released after 72 h in relation to the glucose in the pretreated material.

3. RESULTS AND DISCUSSION

3.1 Magnetite silanization

The physical-chemical characterization of synthesized superparamagnetic magnetite nanoparticles with a mean diameter of 10 nm and saturation magnetization (M_S) of 72.0 emu g^{-1} is well documented in Valenzuela et al. (2010) [14].

Figure 1 displays the FT-IR spectrum of (a) magnetite and (b) magnetite chemically bonded to 3-APTS. The magnetite spectrum shows the characteristic absorption bands of Fe-O bond at 583.05 and 633.75 cm^{-1}. When silanized these absorption bands slightly shifts to higher wavenumbers 584.07 and 634.80 cm^{-1}, respectively, attributed to the formation of Fe-O-Si bonds. Fe-O-OH groups on the surface of magnetite nanoparticles are replaced by Fe-O-Si(O-)$_2$-R. Since -Si(O-)$_2$ is more electronegative than H, the strength of the Fe-O bond is enhanced, producing the shift of the absorption bands [20]. The absorption band at 1129.38 cm^{-1}, assigned to the stretching vibration of the C-N bond; the band at 1029.42 cm^{-1}, due to the stretching vibration of the Si-O bond; the band at 3397.08 cm^{-1}, due to the stretching vibrations of $–NH_2$; and the bands at 1568.04 and 692.92 cm^{-1} corresponding to bending vibrations of the $–NH_2$ group,

confirmed that 3-APTS was chemically bonded to magnetite.

Figure 2 shows magnetic properties for magnetite coated and uncoated with 3-APTS at 300 K. In both cases, the magnetic curves showed no hysteresis loop and they are reversible. Additionally, neither coercivity nor remanence was observed. The saturation magnetization (M_s) for uncoated magnetite and coated were 72.0 emu g^{-1} and 55.9 emu g^{-1}, respectively. These results agree with Yamaura et al. (2004) [21] proving that 3-APTS coating contributes as non-magnetic mass to the total sample volume.

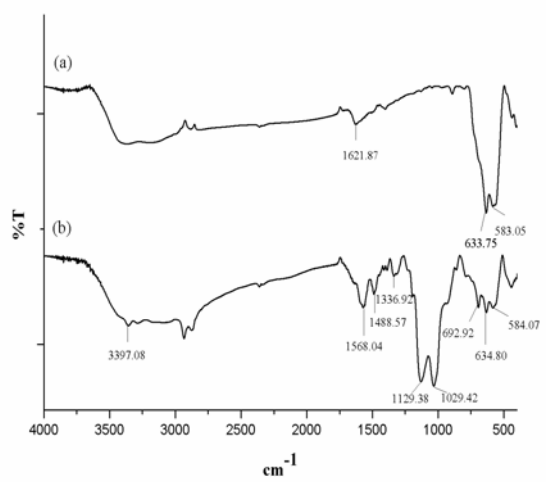

Figure 1: FT-IR spectra of (a) Fe_3O_4 and (b) Fe_3O_4 coated with 3-APTS.

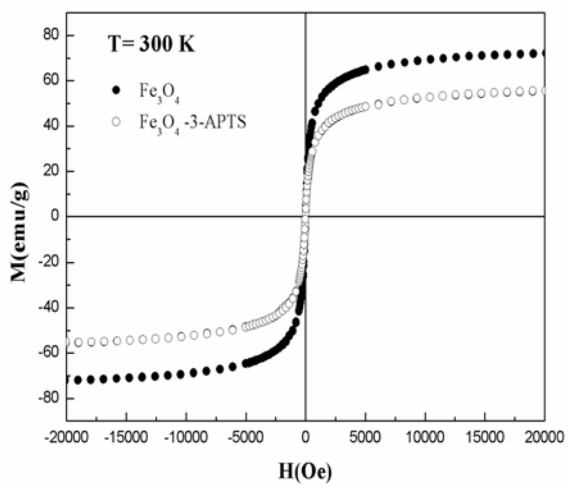

Figure 2: Hysteresis loops of (a) Fe_3O_4 and (b) Fe_3O_4 coated with 3-APTS.

3.2 β-glucosidase Immobilization

Mean concentration of I-βG determined by the Bradford method was 0.10 ± 0.02 mg ml^{-1}. Specific enzymatic activity of I-βG and F-βG was 110.5 ± 1.0 IU mg^{-1} and 112.5 ± 0.5 UI mg^{-1}, respectively.

I-βG hydrolyzed 100% of 2.5 g ml^{-1} cellobiose in 60 min and can be magnetically harvested and reutilized over 45 days without losing its ability to hydrolyze cellobiose. These results indicate that the enzymatic activity was maintained and no denaturation of the immobilized enzyme was observed when stored at 4º C and used for different periods at 50º C.

3.3 Enzymatic Hydrolysis of Pretreated Material

The results of enzymatic hydrolysis of the pretreated materials by cellulase supplemented with I-βG and F-βG and without supplementation are displayed in Figure 3. It can be seen that after 72 h cellulase supplemented with I-βG the mean hydrolysis yields on pulp basis obtained were 76.1%, 83.6% and 75.6% for WS-SE, EG-H and EG-HA, respectively. The I-βG were magnetically recovered and reused twice and the differences in the hydrolysis yields were not significant ($p>0.05$) for WS-SE and EG-HA, whereas the I-βG recycled from EG-H hydrolysis suffer a decrease of 10% in the yield of hydrolysis that can be attributed primarily to the loss of magnetite during the multiple washes after the first recovery.

The sedimentation of I-βG may explain lower hydrolysis yields at 72 h for all cases when compared with the hydrolysis of cellulase supplemented with F-βG.

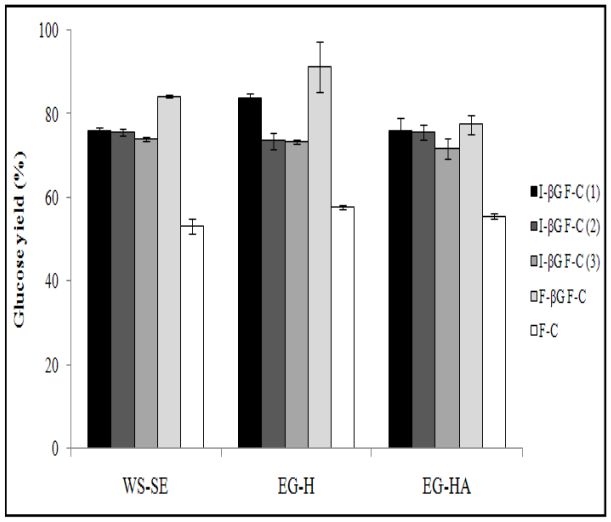

Figure 3: Glucose yields from enzymatic hydrolysis of wheat straw and *Eucalyptus globulus* pulps at 72 h with immobilized β-glucosidase and free cellulase used three times. **I-βG F-C (1), (2), (3):** immobilized β-glucosidase and free cellulase (numbers of use); **F-βG F-C**: free β-glucosidase and free cellulase; **F-C**: free cellulase.

The yield of glucose during enzymatic hydrolysis using cellulase without β-glucosidase supplementation was lower than using supplementation with F-βG or I-βG, increasing the concentrations of cellobiose and glucose (data not shown) that produces end-product inhibition of the endo and exoglucanases components of the cellulase complex.

4. CONCLUSION

Immobilized β-glucosidase on superparamagnetic amino functionalized magnetite nanoparticles was successfully used to supplement cellulase for the saccharification of wheat straw pretreated by steam explosion and *E. globulus* pretreated by hydrothermolysis and hydrotermolysis with alkaline extraction. The results showed that after 72 h the enzymatic hydrolysis by cellulase supplemented with I-βG were lower than the enzymatic hydrolysis with cellulase supplemented with F-βG, a difference that can be attributed to the sedimentation of the enzyme/magnetite support. However, immobilized enzymes were easily recovered using magnets and reused. The reusability of immobilized enzymes on magnetite nanoparticles, in addition to new technologies used to produce cheaper enzymes with higher enzymatic activities provides a good opportunity to reduce the overall cost of the bioethanol production process.

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