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OPTIMIZATION OF ENZYME ACTIVITY OF CELLULASES IMMOBILIZED TO MAGNETIC NANOPARTICLES WITH VARYING FUNCTIONAL GROUP DENSITIES

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Abstract

Cellulases are a collection of enzymes that act synergistically to hydrolyze cellulose and produce glucose, which can be fermented into a renewable energy source; bioethanol. There is a great inclination to develop reusable catalysts that are capable of hydrolyzing cellulose. This could be achieved by immobilizing cellulases to magnetic particles, which can be magnetically collected and reused. Determining the amount of enzyme immobilized, the conformation of immobilized enzymes, and the functional group density on the magnetic nanoparticle (MNP) is essential when surveying a catalytic support modification. Functional group density affects both the amount of enzyme immobilized as well as the immobilized catalysts conformation. The conformation of immobilized enzymes can alter the enzyme's ability to interact with substrate. In the present review, we explored covalently immobilized cellulases adsorbed to MNP supports to optimize varying degrees of functional group densities. The MNP functional group density displayed a positive relationship with the amount of cellulase adsorbed ranging from 130 ± 40 to 320 ± 30 mg cellulase/g MNP, whereas the functional group density was inversely related to the specific activity which ranged from 0.75 ± 0.36 to 0.38 ± 0.05 U/mg enzyme. This work offers an approach to assess enzyme Immobilization and specific activity on MNP's with varying degrees of functional group densities.

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Introduction

There is a critical desire to develop a sustainable source of renewable energy with reduced carbon emissions. In 2021, fossil fuels accounted for ~79% of the energy consumed in the United States; a non-renewable source of energy that is the cause for a significant amount of carbon dioxide emissions.[1] Related to energy in the United States, renewable energy makes up ~12% of energy used, with only 5% of this energy originating from biomass related sources.

Inedible biomass is the most abundant renewable resource and is composed of ~45% cellulose, a polymer of D-glucose linked by β-1,4-glycosidic bonds.[2] Bioethanol derived from cellulose can be a sustainable energy source, but requires extensive processing to hydrolyze the cellulose into glucose prior to fermentation into bioethanol. Cellulose hydrolysis can be performed through chemical or enzymatic methods to yield fermentable sugars. Enzymatic hydrolysis of cellulose has the advantage over non-enzymatic methods due to lower energy input via lower operational temperatures. However, cellulases contribute an appreciable cost to the production of bioethanol which has hindered widespread adoption of enzymatic hydrolysis of cellulose.[3,4] Therefore, an increase in cellulase efficiency would lead to commercialization of bioethanol from cellulose.

One method to increase the cost-effectiveness of enzymes is to immobilize them on solid support materials which facilitates the separation and reuse of the catalyst. Magnetic nanoparticles (MNP) offer desirable traits for enzyme immobilization such as high surface area to volume ratios, enabling a high enzyme immobilization. MNP's also can be rapidly recovered by application of an external magnetic field providing the capability of enzymatic separation. The ease with which MNPs can be separated and reused has led to their study as support for cellulase immobilization.[4]

Two of the most common methods of enzyme immobilization are adsorption and covalent attachment. Adsorption relies on favorable nonspecific interactions between the enzyme and the support. In covalent attachment, a covalent bond is formed between the support and the enzyme. While covalent attachment reduces the possibility for enzyme leeching from the support, covalent attachment may affect the conformation of the immobilized biomolecule; overall affecting the biomolecule activity. Control over the covalent attachment can be obtained by manipulating the chemistry on the surface of the support. A common regent utilized to introduce amine functionality to MNPs is (3-aminopropyl)triethoxysilane (APTES).[5] Varying the APTES concentration during modification can be used to introduce varying degrees of amine functional groups to the MNP.[6]

The surface chemistry/density affects the quantity of enzyme adsorbed, potentially leading to altered biomolecule activity. High densities of immobilized biomolecules sterically hinder the catalysts ability to interact with a substrate, and/or undergo motions required for catalysis.[7,8]

In this work, silica-coated Fe_3O_4 MNPs were modified with a range of [APTES] and subsequently used to immobilize cellulase as shown in **Figure 1**. We observed increased immobilization at high [APTES] ratios while the specific activity was inversely related to [APTES]. The conflicting parameters lead to the most efficient immobilization of cellulase on MNPs modified with moderate [APTES].

Experimental Methods

Materials

Iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) and aqueous glutaraldehyde (50%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Iron (II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), and 3-(aminopropyl)triethoxysilane (APTES) were purchased from Acros Organics (Geel, Belgium). Cellulase from *T. reesei* was purchased from Abnova (Walnut, CA). Copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), sodium acetate, sodium carbonate, sodium bicarbonate, sodium monohydrogen phosphate, sodium dihydro-

gen phosphate, aqueous ammonium hydroxide (29%) and tetraethyl orthosilicate (TEOS) were purchased from Fisher Scientific (Pittsburgh, PA). Sodium bicinchoninate (BCA), carboxymethyl cellulose (CMC) sodium salt (n ~ 500) and hexylamine were purchased from Tokyo Chemical Industry (Portland, USA). The 3,5-dinitrosalicylic acid (DNS) was purchased from Spectrum (New Brunswick, NJ).

Synthesis and Modification of Fe_3O_4 Nanoparticles

Fe_3O_4 nanoparticles were synthesized utilizing a coprecipitation method In brief, a 47 mL solution containing 60 and 30 mM of Fe^{3+} and Fe^{2+} respectively, in 18 MΩ H_2O was heated to 80 °C and degassed with N_2. Following 30 min of degassing, 2.2 mL of NH_4OH was added dropwise and allowed to react for 1 hr. The black product was isolated via magnetic separation then washed using degassed 18 MΩ H_2O three times followed by three subsequent washes with ethanol after which the volume was brought to 47 mL with ethanol to which 2 mL of 200 mM TEOS was added. The hydrolysis of TEOS was initiated by the rapid addition of 5 mL NH_4OH. The solution was immediately placed on a shaker set at 25 °C and 350 RPM and left to react overnight. After modification with TEOS, the MNPs were isolated via magnetic separation then washed with ethanol three times. The amine surface functional groups were introduced to the MNPs by the condensation of APTES. In brief, 30 mg of TEOS modified MNP was added to a scintillation vial containing 10 mL of 3-300 mM APTES in ethanol. The vials were placed on a shaker set at 25 °C and 350 RPM for 12 hr. After amine modification, the MNPs were isolated via magnetic separation then washed three times with ethanol.

Quantitative Analysis of Enzyme Immobilization

Glutaraldehyde was used as an agent to covalently link cellulase and the primary amine from APTES on the MNP surface. APTES MNPs were dispersed at a concentration of 1.0 mg/mL in a solution containing 10 mM phosphate, 20 mM glutaraldehyde at pH 7.4 and reacted on a shaker set at 25 °C and 350 RPM for 30 min. Residual glutaraldehyde was removed from the activated MNPs by washing the MNPs four times with 10 mM acetate buffer pH 5.0. Cellulase in acetate buffer was added to the glutaraldehyde activated MNPs modified at a 1:1 mass ratio of cellulase to MNP.

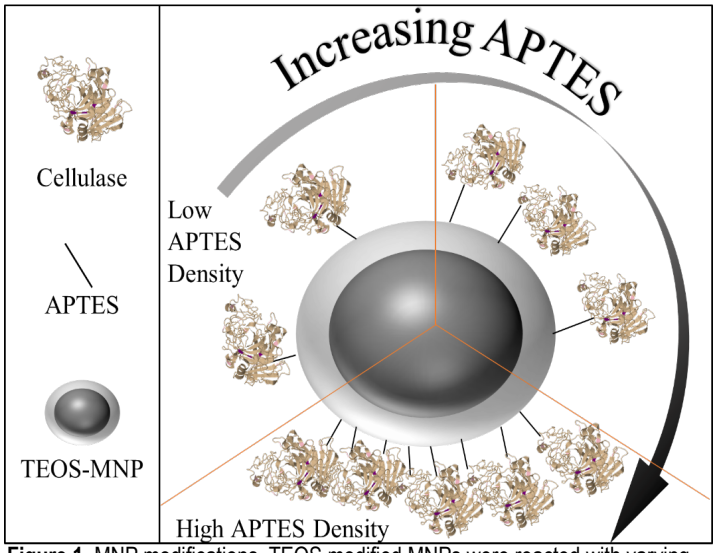

Figure 1. MNP modifications. TEOS modified MNPs were reacted with varying [APTES] and then used for cellulase immobilization. The [APTES] used for MNP modification controlled the cellulase density on the MNP.

The solutions were placed on a shaker set at 25 °C and 350 RPM for 2 hr after which the cellulase immobilized MNPs were washed with acetate buffer three times. The supernatant from each wash was collected and analyzed by the bicinchoninic acid assay (BCA) assay using bovine serum albumin (BSA) as a standard. **Equation 1** shows how the mass of the cellulase immobilized to the MNPs was indirectly quantified by mass balance.

$$Enzyme\ Immobilzed\ =\ C_iV_i - C_sV_S \qquad \textbf{Equation 1}$$

Where C_i is the initial concentration of the cellulase solution, V_i is the initial volume of solution, C_s is the concentration of the supernatant and V_s is the volume of the supernatant.

Quantitative Analysis of Enzyme Activity

The DNS assay was used to determine the activity of the cellulase immobilized MNPs using glucose as a standard. A total of 200 µL of 0.8% (w/v) CMC in 10 mM acetate pH 5.0 was added to 200 µL of cellulase adsorbed MNPs or free cellulase in 10 mM acetate pH 5.0. The samples were placed on a shaker at 50 °C for 30 min after which 200 µL of supernatant was added to 500 µL of DNS solution and boiled for 5 min. The boiled samples were cooled to room temperature, and their absorbance measured at 540 nm. The DNS solution contained 27 mM DNS, 0.5 M NaOH, 0.64 M sodium potassium tartrate, and 25 mM sodium metabisulfite.

Quantitative Analysis of Specific Activity

The specific activity is represents the activity per unit mass of enzyme. The specific activity was determined by **Equation 2.**

$$Specific\ Activity\ of\ the\ Enzyme\ = \frac{U}{M} \qquad \textbf{Equation 2}$$

Where the U represents the activity of the cellulase as quantified from the DNS assay, and M is the mass of cellulase immobilized to the MNPs as determined from the BCA assay.

Results and Discussion.

MNP Synthesis and Modification

The synthesized MNPs were black, and were readily recovered with application of a handheld magnet, leaving a clear supernatant as shown in **Figure 2**. The MNP pellet could be readily dispersed with gentle agitation and could remain suspended 30 minute without any agitation. Preliminary experiments identified MNPs modified with TEOS would precipitate in 15 minutes when void of agitation. Therefore, all modifications and the enzymatic

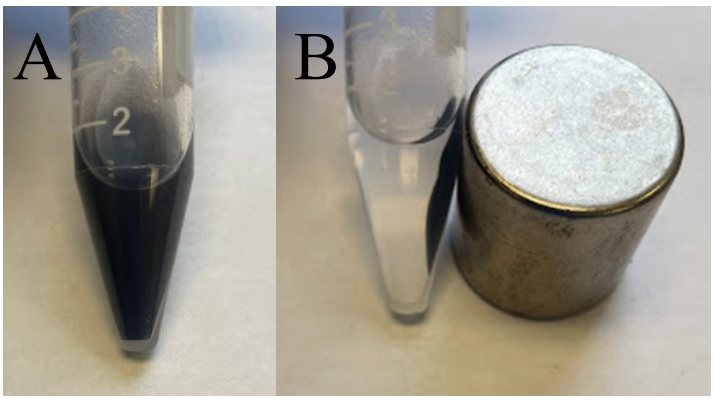

Figure 2. Synthesized MNPs. The as synthesized MNPs (A), and magnetically separated MNPs (B).

hydrolysis were performed on a shaker at 350 RPM to prevent settling.

Cellulase Immobilization

In this work, the primary amines on APTES modified MNPs were activated with glutaraldehyde. Glutaraldehyde acts to covalently link an amine from the MNP to an amine on a biomolecule surface. Once activated, the MNPs were washed to remove unreacted glutaraldehyde and subsequently reacted with cellulase. The quantity of cellulase immobilized to the MNPs ranged from 130 ± 40 to 320 ± 30 mg cellulase/g MNP, while the immobilization efficiency ranged from 16 ± 5 to 48 ± 4% as shown in **Figure 3**. The quantity of cellulase immobilized, and the immobilization efficiency correlated with increases in APTES mole ratio until reaching a level of saturation. The MNPs appear to be saturated with cellulase at the APTES mole ratio of 0.025, 48% immobilization, as no further immobilization was observed with increasing APTES. The immobilization efficiency obtained in this work is lower than other literature reported values that range from 40-80%.[9–11] The reduced immobilization efficiency obtained in this work may be due to the saturation of binding sites on the MNPs, a result that stems from the use of a concentrated enzyme solution used for immobilization. A high immobilization efficiency is essential for the industrial application of reusable cellulases, as cellulases that remain unbound would be unable to be magnetically separated and reused for subsequent cycles.

Cellulose Hydrolysis

The goal of this work was to explore how the APTES mole ratio relates to the overall efficiency of the immobilized catalyst. Cellulases contribute a significant cost to the production of bioethanol from cellulose; therefore, use of cellulase need be optimized. For immobilized cellulases, it is imperative to immobilize abundant quantities of catalyst that are in an active conformation. However, immobilization may alter the conformation of the cellulase which is detrimental in the cellulase activity.[12]

The DNS assay was employed to assess the activity of the immobilized biomolecules which ranged from 90 ± 10 to 150 ± 10 U/g MNP as shown in **Figure 4**. Interestingly, the greatest activity correlated with a moderate APTES mole ratio. To elucidate an explanation to the increased activity at moderate APTES mole ratios, the activity was normalized to the quantity of cellulase immobilized to the MNPs to determine the specific activity of the catalysts.

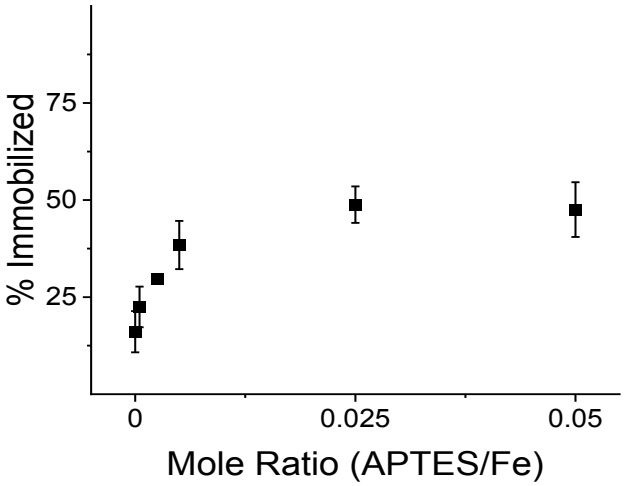

Figure 3 Percent Cellulase immobilized to MNPs. Immobilization was performed in 10 mM acetate pH 5.0 for 2 hours at 25°C. Average ± 1 SD, n = 3 measured in triplicate.

The specific activity of the free cellulase was 0.37 ± 0.06 U/mg while the immobilized cellulases possessed specific activities ranging from 0.75 ± 0.36 to 0.38 ± 0.05 with the greater specific activity correlating with lower APTES mole ratios as shown in **Figure 5**. The MNPs without APTES modification possessed a 48% coefficient of variation in cellulase specific activity. The large variance in this particular group may stem from the biomolecules adopting heterogeneous conformations on the MNPs without APTES. However, the trend in elevated specific activity at low

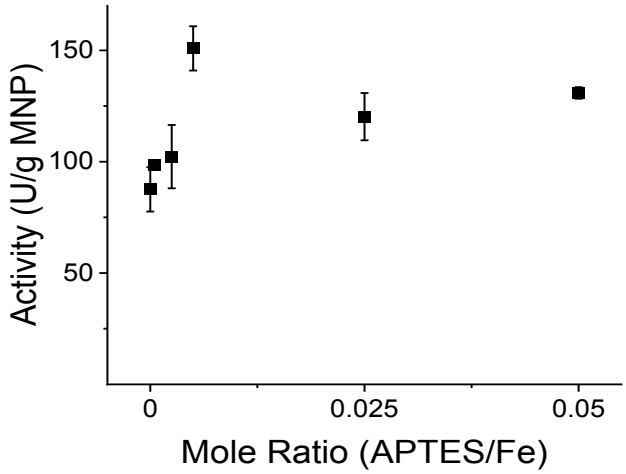

Figure 4. Particle activity. Activity of cellulases assessed using DNS assay at 50°C in 10 mM acetate pH 5.0 and normalized to the mass of MNP. APTES/Fe refers to the mole ratio of functional group density to MNP. Average ± 1 SD, n = 3 measured in triplicate.

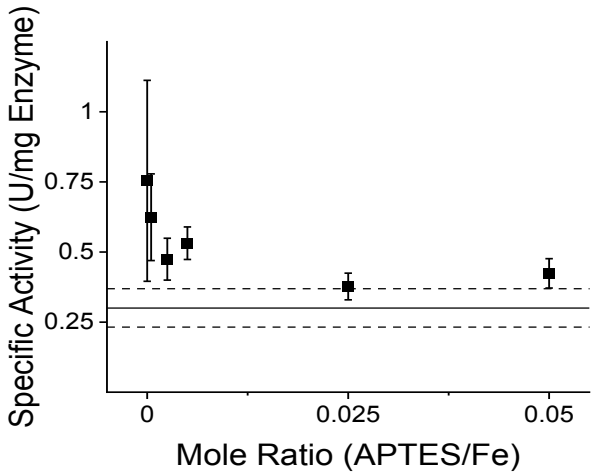

Figure 5. Specific activity of cellulases. Activity of cellulases assessed using DNS assay at 50°C in 10 mM acetate pH 5.0 and normalized to the immobilized amounts. APTES/Fe refers to the mole ratio of functional group density to MNP. Average ± 1 SD, n = 3 measured in triplicate. The solid line indicates the specific activity of the control free cellulase, which was 0.301 U/mg while the dashed lines indicate the 95% confidence interval in the specific activity of the free cellulase. mobilized amounts. APTES/Fe refers to the mole ratio of functional group density to MNP. Average ± 1 SD, n = 3 measured in triplicate. The solid line indicates the specific activity of the control free cellulase, which was 0.301 U/mg while the dashed lines indicate the 95% confidence interval in the specific activity of the free cellulase.refers to the mole ratio of functional group density to MNP. Average ± 1 SD, n = 3 measured in triplicate.

APTES densities is followed by MNPs with APTES modification, as all MNPs with APTES modification possessing less than 25% coefficient of variation. The hyperactivity of the cellulases at low APTES densities may stem from the biomolecule adopting a beneficial conformation on the particle surface. Hyperactivation of immobilized enzymes has been reported previously,[13] most frequently with lipase immobilized to hydrophobic supports.[14] Lipase immobilized to hydrophobic supports adopts a conformation that is more accessible to the substrate therefore possessing increased activity. In this work, cellulase that was immobilized to MNPs with few functional groups possess limited opportunities to form covalent bonds with the surface and therefore form nonspecific interactions with the particle surface leading to the catalyst adopting distorted conformations. In contrast, the enzyme conformation may be retained at high APTES densities as the covalent bonds hold the enzyme conformation in a "rigid" manner, leading enzymes immobilized at high functional groups possessing similar specific activities as the free enzyme. Covalent attachment is a widely studied method to increase the stability of enzymes, as the covalently tethered biomolecule adopts a "rigid" structure which restricts unfolding.[15–17] The origin of the hyperactivity in this work is unknown.

The elevated activity of MNPs with moderate APTES owes to conflicting affects that the APTES mole ratio has on specific activity, and immobilization efficiency. The immobilization efficiency increases with increasing APTES, while the specific activity of the biomolecules is greatest at low APTES ratios meaning one must balance the APTES mole ratio to optimize the amount of catalyst immobilized and its specific activity.

Conclusions

The cost of enzymatic hydrolysis of inedible biomass to glucose prohibit industrial utilization of inedible biomass as a feedstock for bioethanol production. To decrease the financial impact cellulases impose on the hydrolysis of cellulose, they can be immobilized to MNPs and reused for multiple batch cycles. The APTES mole ratio on the MNPs is directly related to the quantity of cellulases immobilized. However, the APTES ratio is inversely related to the immobilized cellulase specific activity. These results lead to optimal activity per unit of MNP at moderate APTES ratios. Industry should therefore immobilize cellulases to particles with moderate functional group densities to decrease the financial impact the enzyme and the support impart on cellulose hydrolysis.

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