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# Magnetic Nanobiocatalysts Based on Immobilized Cellulase

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Biocatalysts based on magnetically responsive supports and the immobilized enzyme - cellulase- demonstrated the high activity in the carboxymethylcellulose (CMC) hydrolysis. Magnetic supports were obtained by two ways: (i) the synthesis of magnetite nanoparticles (NPs) followed by silica shell coating, (ii) the form magnetite NPs by crystallization ones in the pores of mesoporous silica. Physico-chemical methods (low-temperature nitrogen adsorption, scanning electron microscopy, electron-transparent specimens for transmission electron microscopy and magnetic measurements) were utilized for characterization of the structure and surface of the obtained magnetic support. The loading of the enzyme on magnetic supports of both types was approximately the same and amounted to 75-78 %. Cellulase immobilized on magnetic mesoporous silica ($SiO_2$@$Fe_3O_4$@CEL) showed 87 % activity of the free enzyme. The scientific novelty of this work is that the controlled pore size in the mesopore range allows bulky enzyme molecules to be immobilized. The large surface area improves catalytic efficiency by increasing enzyme loading and finely dispersing biocatalyst molecules. The limiting effect of mesopores can improve the stability of the enzyme and its resistance to extreme pH and temperature. However, the use of immobilized cellulase may be limited due to the efficiency of the regeneration process. As a solution to this problem, the use of magnetic nanoparticles incorporated into a mesoporous silica matrix is proposed. The magnetic nanobiocatalyst can be easily removed from the reaction mixture using an external magnet.

## 1. Introduction

Food production, biomass processing, woodworking is necessarily accompanied by the production of waste with a high content of cellulose. The existing industrial thermochemical processes of processing cellulose-containing waste to produce different types of fuels and basic chemical products are carried out at high temperature, pressure using environmentally hazardous corrosive reagents (Califano et al., 2020). The use of biotechnological methods and renewable resources as an alternative to industrial chemistry is of great interest for the development of "green chemistry" and reducing the carbon footprint.

Enzymatic hydrolysis has been put forward as a potential process for biomass conversion. The three main enzymes ("cellulolytic complex enzymes") decomposing cellulose are collectively known as cellulase (CEL). The main function of cellulase includes hydrolysis of polymer cellulose to glucose monomer. Complete degradation of the cellulose molecule will be achieved due to the action of all three enzymes. The first enzyme involved in the decomposition of cellulose is endoglucanase (EG) (1,4-β-D-glucan-4-glucanohydrolase; EC 3.2.1.74), which randomly affects the glycoside bonds inside the cellulose molecule. The reducing or non-reducing ends of cellulose are then further decomposed by exoglucanase (EC 3.2.1.91 and EC 3.2.1.74) to form either cellobiose or glucose. The complete hydrolysis process did not occur without the enzyme β-glucosidase (BGL) (EC 3.2.1.21), which acts on cellodextrins and cellobiose obtained as a result of previous enzyme actions, and is converted into glucose (Chen et al., 2020). Cellulase enzymes act sequentially and synergistically on the cellulose polymer chain, releasing glucose. Further conversion of the resulting glucose yields such valuable platform chemicals as bioethanol, butanol, ethylene glycol, propylene glycol, sorbitol, gluconic acid, levulinic acid, gamma-valerolactone, etc., which are in demand in various industries.

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Porous silica materials are widely used to immobilize enzymes in order to use them as biocatalysts or biosensors. Mesoporous silicates are promising candidates for enzyme immobilization in accordance with the requirements of enzyme carriers, such as large surface area, narrow pore size distribution, well-defined pore geometry, their thermal and mechanical stability and toxicological safety. In addition, the surface of silica substrates can be chemically modified by various functional groups (Costantini et al., 2020). Silicon dioxide is a porous material with a wide surface area consisting of active groups in the form of silanol (-SiOH) and siloxane (Si-O-Si) (Chengmin et al., 2020). Its porosity makes silica act as a high-quality absorbent, in addition, the active silanol (-SiOH) group in silica allows silica to interact with other molecules through hydrogen bonds. When considering the immobilization of enzymes in general and, in particular, cellulase, there are factors that need to be considered in order to maximize adsorption. Firstly, the surface charge of the enzyme and the carrier should be opposite, since electrostatic interactions have a great influence on the adsorption and desorption of the enzyme on the carrier (Devendra et al., 2019). The second is the correspondence between the mesopore size and the molecular diameter of the enzyme; that is, the pore size should be large enough to accommodate the enzyme, but not too large to promote desorption. Finally, the functionalization of the silica surface by hydrophobic groups can also play an important role in the adsorption and desorption of enzymes, since cellulases have a high affinity for hydrophobic surfaces. However, a high load does not always correspond to increased enzyme activity. In (Gaurav et al., 2017) it was studied whether the use of silica for cellulase immobilization, including the effect of contact time and mixing rate during immobilization, affects % immobilization and the activity of immobilized cellulase. In the work of (Haodao et al., 2020), micro- and nanoparticles with a pore size of 9-10 nm and a pore entrance of 5-6 nm were synthesized. They found that cellulase immobilized on nanoparticles has a higher activity than cellulase immobilized on microparticles. The maximum catalytic activity was expressed by cellulase immobilized on nanoparticles modified with triethoxysilane, with the highest loading capacity. Zhang et al. applied a functionalization procedure for covalent immobilization of cellulase on a silica gel substrate. Commercial silicon dioxide with a pore size of 10.6–16.2 nm was used, which decreased to 7.7–10.6 nm after surface functionalization. In addition to its high practicality, the immobilized enzyme also demonstrated high storage stability, retaining 92.4 % of its original activity after storage at 4 °C for 32 days. In the work of (Lawson et al., 2018), two mesoporous silica with different pore sizes of 17.6 nm and 3.8 nm were synthesized. They used these mesoporous materials to immobilize cellulase by pure physical adsorption. It has been shown that cellulose molecules need a conformational change when interacting with the substrate.

The next stage in the development of biocatalyst design was the use of magnetic nanoparticles that can be manipulated using a magnetic field, which makes it possible to effectively separate the biocatalyst from the reaction medium (Lombardi et al., 2022). Iron oxide nanoparticles exhibit superparamagnetic properties, are non-toxic particles that can be synthesized through the co-deposition of iron salts. Silica coating is often used to stabilize magnetic particles in colloidal suspensions, as the coating strongly affects the surface charge. In this context, in the work (Manaenkov et al., 2019), the excellent efficiency of separation and extraction of similar nanoparticles in high-viscosity technological media using a high-gradient magnetic separator was demonstrated. In the work of Mo et al. (2020), the porous biochar was magnetized with -$Fe_2O_3$ by calcination. After chitosan functionalization and glutaraldehyde activation, the resulting chitosan/magnetic porous biochar served as a substrate for cellulase immobilization by covalent binding. The immobilized cellulase had a relatively high retention of activity - 73.0 %. In the analysis for the possibility of reuse, immobilized cellulase showed a retention of activity by 86.0 % after 10 applications. In (Hartmann et al., 2013), galloisite nanotubes were used as a matrix for the immobilization of the cellulase enzyme, which catalyzed the hydrolysis of cellulose into glucose. The magnetic character of the carrier was introduced by fixing iron oxide nanoparticles in situ, on which cellulase was immobilized using aminosilane surface-functional modification. The immobilized cellulase demonstrated excellent stability at elevated temperatures (=60 °C) and storage capacity compared to their free forms. The biocatalyst retained 68.2 % of its original activity after seven consecutive applications.

In this article we report synthesis of the magnetic nanobiocatalysts by the cellulase immobilization of the physical adsorption method on $Fe_3O_4$-containing supports. One sample of the support was carried out through the synthesis of magnetite nanoparticles (NPs) followed by silica shell coating. Another support was obtained in situ of crystallization of $Fe_3O_4$ nanoparticles in the 6 nm pores of commercial mesoporous silica. Magnetic nanobiocatalysts were tested in carboxymethylcellulose (CMC) hydrolysis compared to the free cellulase.

Cellulose enzymes, including cellulase, play an important role in biotechnological processes in the food, cosmetic, detergent, pulp and paper and related industries. The low thermal and storage stability of cellulase, the presence of impurities, enzyme leakage and non-recyclability are major problems in all these processes. These problems can be overcome by creating effective nanobiocatalysts based on cellulase immobilized in the pores of a mesoporous oxide containing magnetite nanoparticles.

## 2. Experimental

### 2.1 Materials

Iron (III) nitrate nonahydrate (98 %), Iron (III) chloride hexahydrate (99 %), ammonium hydroxide solution (28 %), glycerol (≥ 99.5 %), silica gel (99 %, 6 nm, 200-425 mesh), carboxymethylcellulose (CMC) sodium salt low viscosity (C5678), cellulase (Aspergillusniger, 174.9 U/mg), and phosphate buffered saline (PBS buffer) were purchased from Sigma-Aldrich and used as received. Tetraethyl orthosilicate (TEOS, 99 %), were purchased from Fluka and used without purification. Ethylene glycol (EG) (99.5 %), succinic acid (99 %), and urea (99 %) were all purchased from TCI and used without purification. Ethanol was received from Pharmco-Aaper and used as received.

### 2.2 Synthesis of magnetic supports

Synthesis of magnetite nanoparticles coated with silica-shell ($Fe_3O_4$@$SiO_2$)
The synthesis of magnetite NPs was carried out according to the procedure reported elsewhere (Gaurav et al., 2017). In a typical experiment, 0.8109 g of $FeCl_3 \times 12H_2O$ (3 mmol), 0.1181 g of succinic acid (1 mmol), and 1.8018 g of urea (30 mmol) were completely dissolved in 20 mL of ethylene glycol via vigorous stirring. The resultant solution was placed into a Teflon-lined stainless-steel autoclave and heated to 200 °C for 48 h. The reaction was allowed to cool to room temperature and washed three times with ethanol and three times with water. The sample was dried in a vacuum oven at 60 °C overnight. Coating of iron oxide NPs with a silica shell was carried out according to the published procedure (Gaurav et al., 2017). In a typical experiment, 0.19 g of dry NPs were dispersed in 3 mL of ethanol via sonication for 4 h at room temperature. To the dispersed NPs, 0.222 mL of TEOS and 0.633 mL of ammonia hydroxide were added. The reaction was carried out at 40 °C for 12 h. NPs coated with silica were separated from the reaction solution and washed with three times with water and three times with ethanol, using magnetic separation each time. The material was dried in vacuum at 60 °C overnight. Magnetite nanoparticles coated with silica-shell ($Fe_3O_4$@$SiO_2$) were obtained.
Synthesis of magnetite nanoparticles in the pores of mesoporous silica ($SiO_2$@$Fe_3O_4$)
Magnetically separated oxide was obtained in situ by crystallization of $Fe_3O_4$ nanoparticles in the 6 nm pores of commercial mesoporous silica. In a typical experiment, 2.5 g of silica gel and 0.03 mL of the ammonium hydroxide solution (28 %) was stirred in 10 mL of deionized water for 12 h, separated by bench-top centrifugation at 4,000 rpm and then washed five times with water, using centrifugation to remove supernatant. Then treated silica was added to 10 mL of the ethanol solution containing 2.0 g of $Fe(NO_3)_3 \times 9H_2O$ (5 mmol), stirred for 24 h and then allowed to air dry at room temperature upon stirring. The resulting powder was mixed with 70 drops of EG and placed in a ceramic boat, which was then heated in a tube furnace under argon at 350 °C for 6 h with a heating rate of 2 °C/min. After cooling, the sample was ground into a fine powder with a mortar and pestle. The powder was washed five times in acetone using magnetic separation with a permanent magnet and then dried. Magnetite nanoparticles were formed inside the pores of silica ($Si_iO_2$@$Fe_3O_4$).

### 2.3 Synthesis of biocatalyst

The CEL immobilization by adsorption procedure followed the route. As much as 0.25 g of the support was contacted with 10 mL of enzyme solution (0.25 % w/v). The mixture was then homogenized using a shaker with a speed of 100 rpm for a time variation of 60 min. The mixture was filtered to separate the filtrate from the immobilized cellulase.
The TGA curves of all the samples, not reported in this paper, showed a weight loss from 200 °C. It can be attributed of the degradation of organic component, i.e., the progressive deamination, decarboxylation, and depolymerization arising from the breaking of polypeptide bonds for biocatalyst z. The amount of immobilized enzyme was evaluated, subtracting the weight loss of each biocatalyst to the one of bare support in the range between 200 and 800 °C. Then, the immobilization yield of cellulase (YI) was determined according to the following Eq(1):

$$YI = E/E_0 \cdot 100\ \% \quad (1)$$

where E0 is the weight of the enzyme used in the adsorption step and E that of the adsorbed enzyme.
Samples of biocatalysts were designated as$Fe_3O_4$@$SiO_2$@CEL and $SiO_2$@$Fe_3O_4$@CEL.

### 2.4 Enzymatic hydrolysis

The activity of free and immobilized cellulase was assessed by measuring the concentration of glucose produced by the hydrolysis of carboxymethylcellulose sodium salt (CMC), used as substrate (Roth et al., 2016). The specific activity is defined as μmoles of produced glucose over time (min) per gram of immobilized enzyme. 0.1 g immobilized cellulase ($Fe_3O_4$@$SiO_2$/CEL and $SiO_2$@$Fe_3O_4$/CEL) was added to 2 mL 1 %

carboxymethylcellulose in citrate buffer pH 5.5 and hydrolysis process was carried out for 1 h at 125 rpm and 35 °C. The supernatant was analyzed for its reducing glucose content. The specific activity of free cellulase from *Aspergillusniger*and *Trichodermareesei* was 12.6 and 4.38 U/mg.
The relative activity (RA%) of the biocatalyst was calculated considering the immobilization yield of CEL following Eq(2):

$$RA\% = (Cg_{bc}/YI \cdot Cg_{fe}) \cdot 100\ \% \quad (2)$$

where Cgbc is the amount of reducing glucose from biocatalyst (mg/mL), Cgfe is the amount of reducing glucose from free enzyme (mg/mL), YI is the immobilization yield of cellulase.

### 2.5 Characterization

Electron-transparent specimens for transmission electron microscopy (TEM) were prepared by placing a drop of a sample suspension onto a carbon-coated Cu grid. Images were acquired at an accelerating voltage of 80 kV on a JEOL JEM1010 transmission electron microscope. The images were analyzed with an image-processing package ImageJ (the National Institute of Health) to estimate nanoparticle diameters.
For scanning electron microscopy (SEM), samples were drop cast onto the aluminum SEM stubs. The samples were sputter coated using a Polaron Equipment Ltd, SEM Coating Unit E5100, with a gold/palladium target (Au 60 %, Pd 40 %) for 2 min at 20 mA for a coating of approximately 30 nm. They were imaged on a FEI Quanta 600F with the Everhart Thornley detector at an accelerating potential of 10 kV.
Magnetic measurements were performed on a Quantum Design PPMS-14 magnetometer using the system DC measurement capabilities. The sample (50 mg) was placed in a standard gelatin capsule.
Nitrogen adsorption measurements were carried out at liquid nitrogen temperature on an ASAP 2020 analyzer from Micromeritics. Samples were degassed at 100 °C in vacuum. The total surface area was estimated by the Brunauer–Emmett–Teller (BET) method, while the pore size distribution was determined by the Barrett–Joyner–Halenda (BJH) method using desorption.

## 3. Results and discussion

For the synthesis of$Fe_3O_4$NPs by the polyol process ($Fe_3O_4$@$SiO_2$), we used ethylene glycol as both solvent and reducing agent. This procedure is known to generate iron oxide NPs which tend to assemble into NPs [15]. Varying an iron precursor concentration, we were able to vary the NP size. The $Fe_3O_4$@$SiO_2$NPs of 285±71 nm in diameter are shown in Figure 1a. The SEM image of $Fe_3O_4$@$SiO_2$presented in Figure 1b clearly shows that the NPCs are composed of individual NPs of ~ 42-45 nm in diameter. The silica layer is clearly visible on the $Fe_3O_4$@$SiO_2$NPs (Figure 1c) and is approximately 18 nm thick.

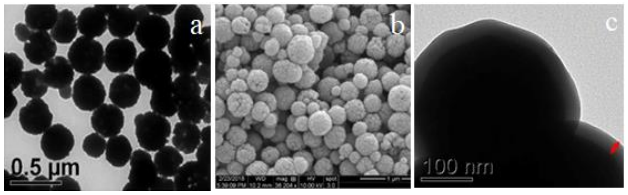


*Figure 1: TEM images (a) SEM image (b) and TEM images (c) of Fe3O4@SiO2. The red arrow in (c) shows a silica layer which has a lower electron density than that of the NPs*

The characteristic nitrogen adsorption-desorption isotherms and pore size distribution for $Fe_3O_4$@$SiO_2$ (Figure 2) shown the BET surface area of this sample is 11.9 $m^2$/g. The isotherms resemble type IV with the H1 hysteresis loop, demonstrating that mesoporosity most probably comes from the spaces between $Fe_3O_4$@$SiO_2$NPs rather than from the inherent porosity of the NPs themselves. At the same time, a close look at the TEM images of the NPs (Figure 1) shows some spaces with a lower electron density within individual NPs. These spaces are most likely internal pores formed during NP clustering and Ostwald ripening within NPs. Because they do not have interconnected pore structure which opens to the NP surface, they do not contribute to the porosity.
For the synthesis of magnetite by crystallization of $Fe_3O_4$ nanoparticles the commercial mesoporous silica was utilized. The liquid nitrogen adsorption data formagnetic NPs in silica pores ($SiO_2$@$Fe_3O_4$) show the adsorption-desorption isotherms represent type IV, commonly observed for mesoporous materials. The BET (Brunauer-Emmett-Teller) method was used to estimate the total surface area. For $SiO_2$@$Fe_3O_4$, it measures 304 $m^2$/g, with pores in 6 nm. A comparison of porosity of the magnetic silica with those of the parent silica sample demonstrates that the total surface area decreases after the magnetite NP formation. It is 30 % for $SiO_2$@$Fe_3O_4$,

indicating there is partial pore blockage. The pore size distributions, however, stay practically unchanged, revealing that the majority of smaller pores are not blocked.
Transmission electron microscopy (TEM) images of $SiO_2$@$Fe_3O_4$ are typical for mesoporous magnetic silicas (Roth et al., 2016). The high resolution (HR) TEM images the magnetite NPs are clearly visible, and their sizes were estimated using ImageJ software. The magnetite NPs in $SiO_2$@$Fe_3O_4$measure 2.4 ± 0.5 nm.

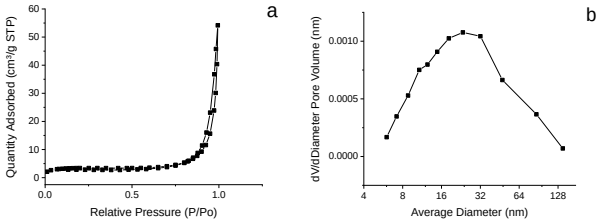


*Figure 2: Nitrogen adsorption-desorption isotherms (a) and pore-size distributions (dV/dD) based on the BJH desorption data using the Faas correction (b) of $Fe_3O_4$@$SiO_2$*

The magnetization curves of $Fe_3O_4$@$SiO_2$(Figure 3) show a mean saturation magnetization of ~ 3.0 emu/g which is consistent with small magnetic NPs. In the synthesis of nanoparticles in silica pores ($SiO_2$@$Fe_3O_4$) the magnetization had a lower value of about 2 emu/g. After each reaction, the catalyst was easily separated from the reaction mixture using a neodymium magnet (which took 30-40 s).

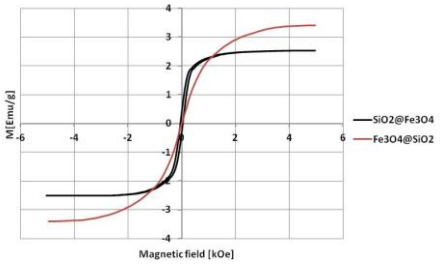


*Figure 3: The magnetization curves of $Fe_3O_4$@$SiO_2$ and $SiO_2$@$Fe_3O_4$*

The activity of the free and immobilized cellulase ($Fe_3O_4$@$SiO_2$@CEL and $SiO_2$@$Fe_3O_4$@CEL) was assessed by measuring the concentration of glucose produced by the hydrolysis of CMC, used as substrate. The specific activity is defined as moles of produced glucose over time (min) per gram of immobilized enzyme. The immobilization of cellulase by the physical adsorption was previously performed and the value of the immobilization yield of cellulose was determined for both samples. It is known that the contribution to physical adsorption is due to the electrostatic interaction between the cellulase and the carrier. In this paper, Si-OH groups are located on the surface for both samples, and enzyme loading is approximately the same 75 % and 78 % for $Fe_3O_4$@$SiO_2$@CEL and $SiO_2$@$Fe_3O_4$@CEL. Figure 4 shows the values of the relative activity of biocatalysts compared to the native enzyme. The relative activity was 71 % and 87 % for $Fe_3O_4$@$SiO_2$@CEL and SiO2@$Fe_3O_4$@CEL.

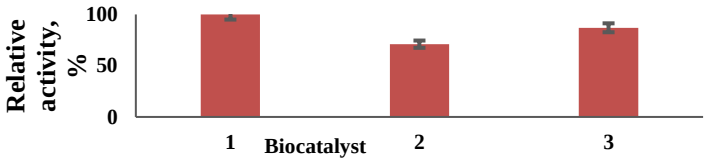


*Figure 4: Relative activity (%) of the different biocatalysts: (1) Free enzyme, (2) $Fe_3O_4$@$SiO_2$@CEL, (3) $SiO_2$@$Fe_3O_4$@CEL*

It is likely that such a difference can be explained by the different structure and difference of the total surface area and porosity of magnetic media. The first carrier, magnetite nanoparticles coated with silica ($Fe_3O_4$@$SiO_2$), has a small total surface area and a large pore size spread (Figure 2). Such pairs are able to accommodate cellulase, whose molecular size is 5.2 x 7.6 x 11.3 nm. At the same time, the conformation of the enzyme can

be different and the catalytic centers are not always available for the substrate. For the second carrier, when magnetite nanoparticles form 6 nm pores of commercial mesoporous silica ($SiO_2$@$Fe_3O_4$), cellulase adsorption is carried out on the surface. The total surface area for ($SiO_2$@$Fe_3O_4$) is almost 30 times larger compared to the $Fe_3O_4$@$SiO_2$ sample. The enzyme that is on the surface turns out to be more accessible and more active in the hydrolysis of CMC.

## 4. Conclusions

The magnetic nanobiocatalysts by the cellulase immobilization of the physical adsorption method on $Fe_3O_4$-containing supports were synthesized. Two methods of magnetic supports synthesis have been utilized: one way was through the synthesis of magnetite NPs followed by silica-shell coating, another one was obtained in situ of crystallization of magnetite NPsin the pores of mesoporous silica. The structure and surface of the obtained magnetic supportshave been characterizedusing the following methods: low-temperature nitrogen adsorption, scanning electron microscopy, electron-transparent specimens for transmission electron microscopy and magnetic measurements. After cellulase immobilization, the enzyme loading for each nano bio catalyst was determined. The value of YI was 75 % and 78 % for $Fe_3O_4$@$SiO_2$@CAO and $SiO_2$@$Fe_3O_4$@CEL. The activity of immobilized cellulase was tested in the in carboxymethylcellulose (CMC) hydrolysis in comparison with the free cellulase. Considering the enzyme content after immobilization, the relative activity was 71 % for the $Fe_3O_4$@$SiO_2$@CEL and 87 % for the $SiO_2$@$Fe_3O_4$@CEL compared to the native enzyme. It is likely that such a difference can be explained by the different structure and difference of the total surface area and porosity of magnetic media. High catalytic activity of $SiO_2$@$Fe_3O_4$@CELcombined with facile magnetic separation and commercially available parent silica support make this nanobiocatalyst promising for successful commercialization.

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