

Nano-Engineered Fe_3O_4 -Cellulase and Fe_3O_4 @C-SO₃H for RSM-Optimized Recyclable Cellulose Hydrolysis

Hussein M. Hussein^{1*}, Bahaa T. Shawky², Hamed Mohamed Abdel Barry², Ibrahim El Tantawy El Sayed²

1. Department of Chemistry, Faculty of Science, Menoufia University, Shebin El Koom - 13829, Egypt.

2. Microbial Chemistry Department, Biotechnology Research Institute, National Research Centre, Cairo, Egypt

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*Corresponding author: dr.husseinibrahim@yahoo.com (Hussein M. Hussein)

Abstract

Magnetic iron oxide nanoparticles are a promising support for the immobilization of enzymatic and acid catalysts for cellulose hydrolysis because of their large surface area, modifiable surface, and ease of magnetic separation. Here, Fe_3O_4 nanoparticles were synthesized via coprecipitation, yielding quasi-spherical particles with a diameter of 15.3 ± 2.7 nm, PDI of 0.311, and zeta potential of -18.7 mV, confirming moderate monodispersity and colloidal stability. These nanoparticles were employed as supports for cellulase immobilization (biocatalyst), as a core for carbon coating, and subsequently sulfonated to produce a magnetically separable solid acid (Fe_3O_4 @C-SO₃H). Fe_3O_4 nanoparticles were prepared by precipitation from $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ in a basic solution, tested for magnetic, color, and dispersion characteristics, and then characterized using spectroscopy and microscopy before being used for enzyme adsorption and carbon coating/sulfonation. The immobilized cellulase on Fe_3O_4 nanoparticles was applied to the hydrolysis of carboxymethyl cellulose (CMC), while Fe_3O_4 @C-SO₃H was used for the hydrolysis of pretreated cellulose. In all cases, glucose was detected using the glucose oxidase-peroxidase (GOD-POD) method, and the reusability of the magnetic materials was evaluated through multiple recycling runs, retaining >80% activity after five cycles, which demonstrated that they maintained high activity over at least five uses. Response surface methodology (RSM) optimization revealed peak glucose yields of 84.93% from cellulose at pH 5, 60°C, 2.5% substrate, and 17.05 mg/g catalyst loading ($R^2=0.9732$), approaching 91.6% of the performance of commercial enzymes (92.7%) with superior pH and temperature stability and recyclability. This study shows that straightforward Fe_3O_4 -based materials can be used to prepare magnetically recoverable and reusable catalysts for converting cellulose into fermentable sugars.

Keywords: Fe_3O_4 nanoparticles, Magnetic nanocatalysts, Cellulase immobilization, Magnetic recyclability, Solid acid catalyst.

1. Introduction

The conversion of lignocellulosic biomass to fermentable sugars is a significant part of a sustainable biofuels and biobased chemicals production process [1]. Cellulose, the major component of lignocellulosic biomass, is a β -1,4-linked glucan polymer with high intra- and intermolecular hydrogen bonding, making it resistant to hydrolysis [2,3]. Cellulase-catalyzed hydrolysis is highly selective, occurs under mild conditions [4], but is expensive and difficult to recycle [5], highlighting the need for immobilized cellulase. Meanwhile, solid acid catalysts for cellulose hydrolysis have also emerged as a promising nonbiological alternative that can

withstand more extreme reaction conditions and be readily recovered [6,7].

As the world bioethanol consumption is estimated to reach 132 billion liters by 2030 and lignocellulosic feeds account for less than 5% of the current output [8], economic feasibility will depend on catalyst recyclability (>80 percent activity retention after many cycles) and glucose recovery (>85 percent). Fe_3O_4 nanoparticles overcome both of these limitations through superparamagnetic recoverability (20 s separation) [9], 200-300 m²/g surface area of 200-300 m²/g capable of loading 3-5 times more enzyme (15-25 mg/g), and variable surface chemistries that can be

tuned to combine enzyme and acidic activity [10,11].

Fe_3O_4 nanoparticles are desirable supports and catalyst cores because of their small size, high surface area, and high magnetizability, which allows for efficient separation from the reaction mixture with a magnet [12]. Immobilization of cellulase on Fe_3O_4 and other nanostructures has been demonstrated to improve thermal and pH stability and enable reuse over several cycles with high activity retention [13,14]. Similarly, Fe_3O_4 -based core-shell magnetic solid acids, such as $\text{Fe}_3\text{O}_4@\text{C-SO}_3\text{H}$, have been reported as effective catalysts for cellulose hydrolysis, exhibiting both acidity and magnetically recoverable properties [15].

This study evaluates two Fe_3O_4 -based catalytic systems: (1) physically immobilized cellulase for CMC hydrolysis under mild conditions (pH 5, 50°C) and (2) a carbon-sulfonated Fe_3O_4 solid acid for cellulose depolymerization. The $\text{Fe}_3\text{O}_4@\text{C-SO}_3\text{H}$ system achieved an RSM-optimized glucose yield of 84.9%, corresponding to 91.6% of the yield obtained with commercial cellulase under comparable conditions. Both magnetic catalytic systems retained more than 80% of their initial activity after five cycles, indicating their potential for catalyst recovery and reuse.

In this work, we report two different Fe_3O_4 -based catalysts for cellulose conversion. Fe_3O_4 nanoparticles prepared by coprecipitation are used as supports to immobilize cellulase, yielding a magnetically separable biocatalyst for hydrolysis of CMC. The same Fe_3O_4 nanoparticles are also used as a magnetic core for the preparation of a magnetic solid acid, $\text{Fe}_3\text{O}_4@\text{C-SO}_3\text{H}$, via carbon shell coating and sulfonation for cellulose hydrolysis. We present the preparation and characterization of the Fe_3O_4 core and its derived materials, the catalytic activity in cellulose or CMC hydrolysis, and the recyclability of the two catalysts studied.

2. Materials and Methods

2.1. Materials

The sources of iron used for the preparation of Fe_3O_4 were ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) and ferrous chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$). A precipitating agent solution of ammonia hydroxide (NH_4OH) (0.1 M) and deionized water was used. Cellulase enzymes were dissolved in citrate buffer (pH 5), and carboxymethyl cellulose (CMC) was used as the soluble substrate for hydrolysis. In the preparation of solid acid, sugars (glucose) were employed as the carbon source; concentrated sulfuric acid (H_2SO_4) in an organic solvent was used to supply

the acid groups. The amount of glucose was determined by the glucose oxidase-peroxidase (GOD-POD) method at 505 nm, and in some experiments, glucose test strips were used for preliminary screening.

2.1.1. Preparation of Cellulose Substrate

The cellulose substrate used for hydrolysis experiments was extracted from corn cobs via pretreatment with the biocompatible ionic liquid choline lysinate ([Ch][Lys]), following the response-surface-optimized protocol described elsewhere. Briefly, corn cobs were pretreated with 18 wt% [Ch][Lys] at 100°C for 3 hours, achieving $78.63 \pm 0.82\%$ lignin removal and yielding a cellulose fraction with 97.85% enzymatic digestibility, consistent with the selective disruption of lignin-carbohydrate complexes and reduced structural recalcitrance of the recovered cellulose. Amino acid-derived IL anions, such as lysinate, have been reported to favor selective delignification while largely preserving the polysaccharide fraction, distinguishing their mode of action from acetate- or chloride-based ILs [16]. This pretreatment history is relevant to the hydrolysis performance reported below (Section 3.4).

2.2. Synthesis of Fe_3O_4 Nanoparticles

Fe_3O_4 nanoparticles were prepared by the coprecipitation of $\text{Fe}^{2+}/\text{Fe}^{3+}$ ions in an alkaline solution. A $\text{Fe}^{2+}/\text{Fe}^{3+}$ molar ratio of 1/2 was achieved by dissolving 1.3 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 0.478 g of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ in 12 mL of deionized water with stirring. [17]. This solution was stirred at room temperature, and 0.1 M NH_4OH solution was slowly added with vigorous stirring while maintaining the pH of the solution around 10–12 for the formation of magnetite. The suspension was stirred for 30–60 minutes, resulting in a black precipitate of Fe_3O_4 nanoparticles. The suspension was then centrifuged at 10,000 rpm for 10–20 minutes, washed several times with deionized water and ethanol to remove impurities, and subsequently dried at room temperature [18].

2.3. Characterization of Fe_3O_4 Nanoparticles

The magnetic properties of the dry Fe_3O_4 nanoparticle powder were qualitatively determined by exposure to a permanent magnet, which caused the particles to move toward the magnet [19]. The color and dispersibility of the nanoparticles were qualitatively evaluated by suspending them in water using gentle sonication, producing a black suspension that aggregated in a magnetic field and dispersed when the magnet was removed. Ultraviolet-visible (UV-Vis) spectroscopy was used to measure the absorption spectrum in the

200–800 nm range, where Fe_3O_4 nanoparticles exhibit strong, broad absorption in the visible region typical of iron oxide nanoparticles. The Fe_3O_4 nanoparticle powder was analyzed via Fourier transform infrared (FTIR) spectroscopy to identify Fe–O vibrations ($550\text{--}600\text{ cm}^{-1}$) and potential surface hydroxyl or organic groups. Scanning electron microscopy (SEM) was employed to determine particle shape, size (tens of nanometers), and aggregation. Additionally, dynamic light scattering (DLS) and zeta potential measurements were performed to assess the physicochemical properties of the synthesized nanoparticles [20].

2.4. Preparation of Cellulase– Fe_3O_4 Biocatalyst

The preparation of the cellulase biocatalyst involved immobilizing cellulase onto 50 mg of Fe_3O_4 nanoparticles in 5–10 mL of cellulase solution (1–5 mg/mL) in phosphate buffer (pH 5). The mixture was gently shaken at room temperature overnight to ensure complete adsorption. After incubation, the particles were separated magnetically and extensively washed with buffer, while FTIR measurements were used to confirm the presence of the enzyme on the Fe_3O_4 surface [21].

2.5. Preparation of Fe_3O_4 @C and Sulfonated Magnetic Solid Acid

For the magnetic solid acid, Fe_3O_4 nanoparticles were initially coated with a carbon precursor. Fe_3O_4 nanoparticles were suspended in a glucose solution to form a slurry. This was dried to yield an Fe_3O_4 -organic composite powder. The powder was heated at $300\text{--}400\text{ }^\circ\text{C}$ in a hot air oven to convert the organic component into carbon and to form a Fe_3O_4 @C core-shell structure (Fe_3O_4 core with a thin carbon shell). Sulfonation was performed by dispersing Fe_3O_4 @C in concentrated H_2SO_4 under ice-cooled conditions and stirring for 30–60 minutes. It was then heated in a loosely capped vessel at 80°C for 6 hours to graft sulfonic acid groups and enhance the stability of the carbon shell. Subsequently, the product was cooled, carefully diluted with water, magnetically separated, washed with water to neutral pH, and dried to produce Fe_3O_4 @C- SO_3H with significant magnetization [22].

2.6. Enzymatic Hydrolysis of CMC with Immobilized Cellulase– Fe_3O_4

The activity of cellulase– Fe_3O_4 was tested with a 3 wt% CMC solution in buffer. Fifteen milligrams of immobilized cellulase– Fe_3O_4 were suspended in 10 mL of 3% CMC solution in buffer (pH 5) and shaken at 150 rpm at $50\text{ }^\circ\text{C}$ for 1–24 hours [23]. The reaction products were separated from the

magnetic biocatalyst using a magnet, and the glucose content was measured by the GOD-POD method at 505 nm [24]. The enzyme activity could be expressed in units, with 1 unit defined as the amount of enzyme that releases $1\text{ }\mu\text{mol}$ of glucose equivalents per minute under the assay conditions [25]. CMC was chosen as the substrate for this initial enzyme benchmarking step due to its complete solubility in water, allowing rapid and reproducible activity quantification independent of substrate crystallinity or mass-transfer effects that could otherwise complicate direct comparison; by contrast, the solid acid catalyst was evaluated directly on real pretreated cellulose (Section 2.1.1, 2.7) to assess performance on an authentic lignocellulosic substrate.

2.7. Hydrolysis of Pretreated Cellulose with Fe_3O_4 @C- SO_3H

The pretreated cellulose fraction was hydrolyzed in a 50 mL Erlenmeyer flask. Cellulose substrate with different concentrations (1–5%, w/v) and various concentrations of nanozyme (5–25 mg/g substrate) were dissolved in acetate buffer (50 mM, pH 5.0). The reaction flasks were maintained in a shaker incubator at 150 rpm. Samples were collected at various time points (0, 6, 12, 24 hours) and centrifuged at 10,000 rpm for 5 minutes. The glucose concentration in the supernatants was measured [26]. Glucose yield (% w/w) was calculated relative to the theoretical maximum glucose yield obtainable from the cellulose substrate, accounting for the stoichiometric mass increase associated with hydrolytic water addition during glycosidic bond cleavage:

$$\text{Glucose Yield (\%)} = \frac{\text{Mass of glucose produced}}{\text{Mass of cellulose substrate} \times 1.111} \times 100$$

Where the conversion factor of 1.111 (180/162) accounts for the addition of one water molecule (18 g/mol) per anhydroglucose unit (162 g/mol) converted to free glucose (180 g/mol) during hydrolysis.

To control for potential background interference from leached iron ions or residual catalyst material with the GOD-POD colorimetric assay, blank samples containing the respective catalyst (Fe_3O_4 -cellulase or Fe_3O_4 @C- SO_3H) at the same loading, in reaction buffer without cellulose substrate, were run in parallel under identical conditions for each experimental run. The absorbance of these blanks was subtracted from the corresponding sample absorbance prior to glucose quantification, correcting for any non-specific

background signal arising from the magnetic catalyst itself.

As a comparison, commercial enzymes, namely Celluclast 1.5L (cellulase) and Novozyme 188 (β -glucosidase), were applied at 15 FPU/g substrate and 30 CBU/g substrate, respectively, under similar conditions.

2.7.1. Optimization of Hydrolysis Conditions

To determine the optimal hydrolysis conditions, we explored the influence of pH (3.0–7.0), temperature (30–70°C), substrate concentration (1–5%, w/v), and nano enzyme (5–20 mg/g substrate) on the yields. The optimization was performed using a central composite design statistical model.

2.8. Statistical Analysis

Triplicate measurements were taken, and the results are presented as mean $\pm$ SD. Data analysis involved one-way ANOVA followed by a Tukey post hoc test, with significance set at $p < 0.05$ using Design Expert software (version 11.0) [27].

Triplicate measurements refer to technical replicates (three aliquots from the same reaction batch, processed and measured in parallel under identical conditions), reflecting analytical measurement precision rather than independent biological or batch-to-batch variability.

2.9. Reusability Tests

The reusability of the immobilized cellulase - Fe_3O_4 and Fe_3O_4 @C-SO₃H solid acid catalyst was also tested. Following each hydrolysis, the magnetic catalyst was separated from the reaction mixture using a magnet, and the supernatant was removed for sugar analysis within about 10 seconds. The catalyst was then washed and reused for the next run with fresh reaction substrate. Five cycles were conducted, and the remaining activity or catalytic efficiency was calculated as a percentage of the original activity.

3. Results and Discussion

3.1. Formation and Properties of Fe_3O_4 Nanoparticles

The simultaneous precipitation of Fe^{2+} and Fe^{3+} ions in the presence of an alkali resulted in a black precipitate that was strongly magnetic (attracted to a permanent magnet), suggesting the formation of magnetic Fe_3O_4 nanoparticles. The particles dispersed readily in water to form a dark suspension that completely separated and re-dispersed under an applied magnetic field, consistent with good redispersibility and high magnetization. The UV-Vis spectra of the nanoparticles displayed strong absorption in the visible region, as expected for iron oxides. FTIR spectra (Figure) showed stretching bands of Fe–O

at 570–580 cm^{-1} and bands that could be assigned to hydroxyl groups or residual organics on the surface, while SEM images revealed nearly spherical, tens of nanometers-sized particles with a degree of aggregation. The Fe_3O_4 nanoparticles were found to be quasi-spherical, with a diameter of 15.3 ± 2.7 nm (Figure).

3.2. Characterization of Synthesized Nanozyme

3.2.1. Morphology, Size Distribution and Surface properties

The SEM images of the synthesized nanozyme are presented in Figure . The Fe_3O_4 nanoparticles exhibited a quasi-spherical morphology with an average diameter of 15.0 nm.

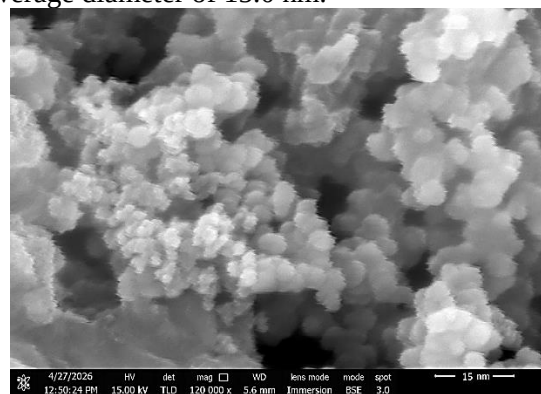


Figure 1. Scanning electron microscope SEM image for produced nano particles

Dynamic light scattering (DLS) and zeta potential measurements were used to assess the physicochemical properties of the synthesized nanoparticles. The DLS analysis (Figure) indicated that the population consisted of a monodisperse system with a narrow size distribution, averaging around 15.3 ± 2.7 nm. This demonstrates successful synthesis with good control over particle size, resulting in a homogeneous nanoparticle system. The polydispersity index (PDI) was calculated as 0.311, indicating a moderately polydisperse but acceptable size distribution. This PDI value aligns with the effectiveness of the synthesis method in producing nanoparticles with reproducible sizes. Zeta potential measurements (Figure) revealed a surface charge of -18.7 mV. This negative charge is sufficient to generate electrostatic repulsion between particles, preventing aggregation in aqueous suspension and contributing to colloidal stability. The symmetry of the surface properties in the sample population is further confirmed by the sharp and distinct peaks. The small size distribution also reflects the reproducibility of the synthesis process, which is important for repeated applications. These physicochemical properties are consistent with values reported in recent literature. Shah et al. (2025) synthesized Fe_3O_4 nanoparticles

via co-precipitation, with an average diameter of 22.76 ± 0.2 nm, and confirmed superparamagnetic behavior through M–H hysteresis loops [28]. Zhou et al. (2023) reported that co-precipitation-based synthesis of Fe_3O_4 reliably yields nanoparticles with PDI values in the range of 0.08–0.15. Although the PDI of 0.31 obtained in the present study is somewhat higher than this range, it remains within the broadly accepted limit for iron oxide nanoparticles prepared by co-precipitation without additional size-selective purification ($\text{PDI} \leq 0.3\text{--}0.4$) [12], and compares favorably with Rani et al. (2025), who reported PDI values of 0.33–0.56 for similarly synthesized coprecipitation-derived iron oxide nanoparticles [29]. The zeta potential of -18.7 mV indicates a degree of colloidal stability, though below the ± 30 mV threshold typically associated with strong electrostatic stabilization. Nanoparticles in this range can still be practically stable through additional stabilization mechanisms, such as steric stabilization from surface hydroxyl groups. Consistent with this, the Fe_3O_4 nanoparticles remained readily redispersible after magnetic separation, with no visible aggregation observed throughout the experiments, aligning with recent characterization studies on Fe_3O_4 by Hirani B. et al. [19].

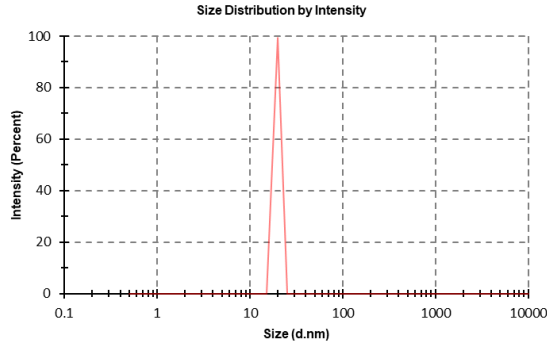


Figure 2. Zeta size for nano particles

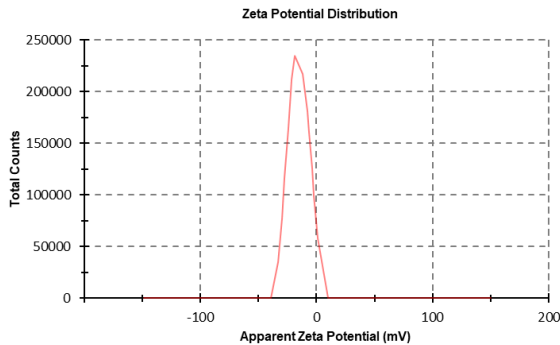


Figure 3. Zeta potential of nanoparticles

Table 1. Physical properties of synthesized nanozyme.

Nano enzyme	Morphology	Average size (nm)	Zeta potential (mV)	PDI
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Fe_3O_4	Spherical	15.3 ± 2.7	-18.7 ± 1.5	0.311
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The FTIR spectrum of the Fe_3O_4 nanoparticles without coating exhibits a relatively simple spectral profile, typical for an iron oxide structure. The most diagnostic feature is the strong vibration at $570\text{--}580\text{ cm}^{-1}$, corresponding to Fe–O stretching associated with the Fe_3O_4 spinel structure. The broad absorption around 3400 cm^{-1} is attributed to the stretching vibration of surface hydroxyl (–OH), while the weaker band around 1620 cm^{-1} is associated with surface –OH bending. These spectral features are consistent with the formation of Fe–O-containing iron oxide nanoparticles bearing surface hydroxyl groups (figure 4a). The FTIR spectrum of the $\text{Fe}_3\text{O}_4\text{ @C-SO}_3\text{H}$ catalyst (figure 4b) exhibits absorption bands consistent with carbon coating and sulfonation of the Fe_3O_4 core. A band at 1711 cm^{-1} is attributed to C=O stretching of carbonyl/carboxylic groups formed during carbonization of the carbon precursor. The band at 1570 cm^{-1} corresponds to the skeletal C=C stretching vibration of polycyclic aromatic carbon sheets, confirming the formation of the amorphous carbon shell around the Fe_3O_4 core. Bands at 1269 and 1202 cm^{-1} are assigned to the asymmetric O=S=O stretching vibration of the sulfonic acid (– SO_3H) group, while the band at 1135 cm^{-1} corresponds to the symmetric O=S=O stretching vibration, together supporting the presence of surface sulfonic acid functionalities onto the carbon shell surface. The bands at 995 and 948 cm^{-1} are attributed to C–O and S–O stretching vibrations, respectively. Importantly, the Fe–O stretching vibration is retained at 570 cm^{-1} (with additional lattice vibrations at 505 and 425 cm^{-1}), consistent with retention of the magnetic spinel core structure following the carbon coating and sulfonation steps. Notably, the broad N–H/O–H stretching band ($\sim 3300\text{--}3400\text{ cm}^{-1}$) and the amide I/II bands ($\sim 1650/1530\text{ cm}^{-1}$) characteristic of the immobilized cellulase spectrum (Section 3.2, Cellulase- Fe_3O_4) are absent from the $\text{Fe}_3\text{O}_4\text{ @C-SO}_3\text{H}$ spectrum, confirming that the two catalyst preparations are chemically distinct and that no residual enzyme/protein contamination is present in the solid acid catalyst. Collectively, these spectral features support the formation of a magnetically separable, Brønsted-acidic solid catalyst bearing surface-associated sulfonic acid functionalities.

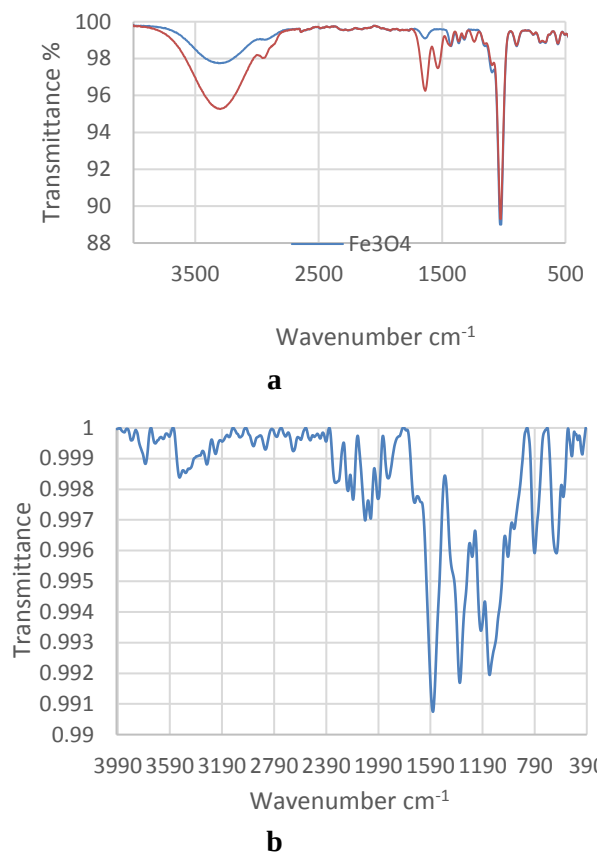


Figure 4. a) FT-IR of Fe_3O_4 NPs and Immobilized Cellulase- Fe_3O_4 , b) FT-IR of Fe_3O_4 @C- SO_3H catalyst

3.3. Immobilized Cellulase- Fe_3O_4 for CMC Hydrolysis

The FTIR spectrum of Cellulase- Fe_3O_4 shows several new and strong absorption peaks compared to the Fe_3O_4 NPs spectrum, confirming the successful immobilization of cellulase enzyme molecules on the surface of Fe_3O_4 nanoparticles. The most prominent feature of the Cellulase- Fe_3O_4 spectrum is the broad absorption band at approximately 3300 cm^{-1} , which can be attributed to the N-H stretching vibrations of amine groups ($-\text{NH}_2$) present in the protein backbone of cellulase, known to play a major role in enzyme binding to solid supports. Additionally, the amide I band ($\text{C}=\text{O}$ stretching of peptide bonds, $\sim 1650\text{ cm}^{-1}$) and amide II band (N-H bending coupled to C-N stretching, $\sim 1530\text{ cm}^{-1}$) appear as new peaks, indicating characteristic features of the enzyme protein structure. The broad C-H stretching band around $2900\text{--}3000\text{ cm}^{-1}$, which is absent in the Fe_3O_4 spectrum, further confirms the presence of organic biomolecular content from the enzyme coating. The presence of the Fe-O peak around 580

cm^{-1} in the spectrum of cellulase- Fe_3O_4 also confirms that the magnetic core structure remained unchanged after immobilization; the appearance of these new bands collectively indicates that significant structural changes occurred during the synthesis of the nano-enzyme, thereby validating the successful loading of cellulase onto the Fe_3O_4 surface (Figure).

Physical adsorption of cellulase onto Fe_3O_4 in buffer resulted in the formation of magnetically separable biocatalysts with considerable activity for CMC hydrolysis. The cellulase- Fe_3O_4 particles could be easily separated from the reaction solution within seconds via magnetic separation and reused for multiple rounds of CMC hydrolysis, thus reducing cellulase waste compared to free enzymes. According to Kazeem et al. [30], cellulase immobilized on Fe_3O_4 -based materials may exhibit enhanced thermostability (with the optimum temperature shifting from 50 to 60°C) and improved pH stability relative to the free enzyme. The immobilized cellulase- Fe_3O_4 biocatalyst produced a 71.92% glucose yield from CMC hydrolysis, compared with 83.50% obtained using the Fe_3O_4 @C- SO_3H solid acid catalyst under identical pH and temperature conditions (pH 5, 50°C) but with pretreated cellulose as the substrate, reflecting the different substrate systems targeted by each catalyst as described in Sections 2.6 and 2.7.

3.4. Fe_3O_4 @C- SO_3H Solid Acid for Cellulose Hydrolysis

A carbon shell coating and sulfonation of Fe_3O_4 led to a core-shell Fe_3O_4 @C- SO_3H catalyst with high Brønsted acidity and magnetic properties. The carbon coating, derived from saccharides or natural polymers, not only offered a large surface area and supported the Fe_3O_4 but also introduced $-\text{SO}_3\text{H}$ groups through sulfonation of the carbon coating, which served as acid sites for cellulose hydrolysis. The Fe_3O_4 @C- SO_3H catalysts have been reported to achieve cellulose conversions of 50% and glucose selectivity of over 50% at 140°C in water as solid acids [31]. The glucose concentrations indicated that the Fe_3O_4 @C- SO_3H catalyst remained active after reuse, and the magnetic properties of the catalyst remained unchanged, suggesting that the catalyst structure was stable under hydrothermal conditions. The catalytic performance of the Fe_3O_4 @C- SO_3H nanozyme developed here compares favorably with the literature. Liu et al. (2024) reported that a magnetic solid acid, Fe_3O_4 @PS- SO_3H , achieved a maximum glucose yield of only 64.14% from cellulose

hydrolysis under harsher conditions of 140°C for 10 hours, requiring a 1:1 acid-to-substrate ratio, despite maintaining good catalytic activity over five recycling cycles [32]. This yield is substantially lower than the 83.50% glucose yield achieved in the present work (at the center-point condition of the RSM/CCD design matrix—prior to statistical optimization of the model) under considerably milder conditions (50°C, pH 5, atmospheric pressure), demonstrating the high apparent hydrolysis performance of the $\text{Fe}_3\text{O}_4@\text{C-SO}_3\text{H}$ nanozyme developed here (Figure). Yang et al. (2021) reviewed bioinspired cellulase-mimicking solid acid catalysts and reported that most carbon-based sulfonated systems achieve glucose yields in the range of 4–68% from cellulose [6], a range that the present catalyst substantially exceeds. Kim et al. reported hydrolysis of ball-milled cellulose to approximately 90% conversion and approximately 85% total sugar selectivity within 20 minutes of reaction time using supercritical CO_2 (150°C, 100–300 bar) in the presence of oxidized carbon catalysts, which is not only energy intensive but also requires specialized high-pressure equipment [33], further highlighting the practical advantage of the present $\text{Fe}_3\text{O}_4@\text{C-SO}_3\text{H}$ nanozyme system, which reaches 84.93% glucose yield at atmospheric pressure and mild temperature (60°C, pH 5).

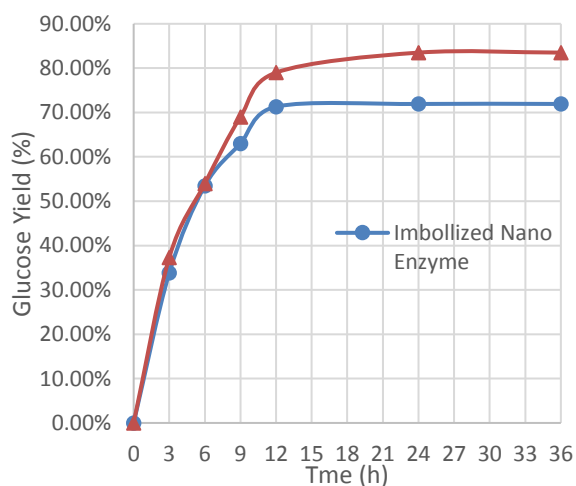


Figure 5. Glucose yield (%) from hydrolysis using Immobilized nanozyme vs $\text{Fe}_3\text{O}_4@\text{C-SO}_3\text{H}$ solid acid catalyst

3.5. Optimization of Hydrolysis Conditions Using RSM

3.5.1. Model Fitting and Statistical Analysis

RSM/CCD optimization was performed exclusively for the solid acid catalyst ($\text{Fe}_3\text{O}_4@\text{C-SO}_3\text{H}$); the immobilized enzyme nanozyme (Fe_3O_4 -cellulase) was evaluated under fixed

standard conditions solely for benchmarking and comparison purposes and was not subjected to full RSM optimization [34]. Table 1 shows the experimental design and the responses (glucose yield %) of $\text{Fe}_3\text{O}_4@\text{C-SO}_3\text{H}$ solid acid catalyst as an example.

Table 1. Central composite design matrix and experimental results for cellulose hydrolysis using $\text{Fe}_3\text{O}_4@\text{C-SO}_3\text{H}$ solid acid catalyst.

Run	pH (X_1)	Temperature (X_2 , °C)	Substrate loading (X_3 , %)	Solid Acid Nano enzyme loading (X_4 , mg/g)	Glucose yield (Y_1 , %)
1	4.0	40	2	10	48.7
2	6.0	40	2	10	57.6
3	4.0	60	2	10	62.3
4	6.0	60	2	10	71.5
5	4.0	40	4	10	45.8
6	6.0	40	4	10	54.1
7	4.0	60	4	10	58.7
8	6.0	60	4	10	67.2
9	4.0	40	2	20	55.6
10	6.0	40	2	20	64.3
11	4.0	60	2	20	70.1
12	6.0	60	2	20	79.8
13	4.0	40	4	20	52.3
14	6.0	40	4	20	60.5
15	4.0	60	4	20	65.7
16	6.0	60	4	20	74.9
17	3.0	50	3	15	37.2
18	7.0	50	3	15	56.4
19	5.0	30	3	15	42.6
20	5.0	70	3	15	67.8
21	5.0	50	1	15	75.3
22	5.0	50	5	15	58.9
23	5.0	50	3	5	52.1
24	5.0	50	3	25	78.5
25	5.0	50	3	15	81.3
26	5.0	50	3	15	82.7
27	5.0	50	3	15	80.8
28	5.0	50	3	15	83.5
29	5.0	50	3	15	82.1
30	5.0	50	3	15	81.9

The experimental results were modeled with a quadratic polynomial equation, and the significance of the model was checked by ANOVA. presents the representative ANOVA results for cellulose hydrolysis with $\text{Fe}_3\text{O}_4@\text{C-SO}_3\text{H}$ solid acid catalyst.

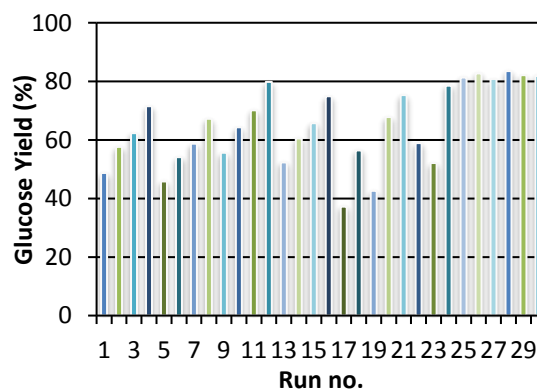


Figure 6. CCD Glucose Yields per Run (Table 2)

Table 2. ANOVA results for the response surface quadratic model for cellulose hydrolysis using Fe₃O₄@C-SO₃H solid acid nanozyme.

Source	Sum of squares	df	Mean square	F-value	p-value	Significance
Model	4976.00	14	355.43	38.87	< 0.0001	significant
A-pH	142.27	1	142.27	15.56	0.0013	significant
B-Temperature	4.36	1	4.36	0.4772	0.5002	
C-Substrate loading	110.10	1	110.10	12.04	0.0034	significant
D-Nano enzyme loading	309.43	1	309.43	33.84	< 0.0001	significant
AB	0.3906	1	0.3906	0.0427	0.8390	
AC	0.3306	1	0.3306	0.0362	0.8517	
AD	0.0506	1	0.0506	0.0055	0.9417	
BC	0.8556	1	0.8556	0.0936	0.7639	
BD	1.16	1	1.16	0.1264	0.7272	
CD	0.2756	1	0.2756	0.0301	0.8645	
A²	1878.66	1	1878.66	205.46	< 0.0001	significant
B²	1046.22	1	1046.22	114.42	< 0.0001	significant
C²	281.05	1	281.05	30.74	< 0.0001	significant
D²	365.63	1	365.63	39.99	< 0.0001	
Residual	137.16	15	9.14			
Lack of Fit	132.48	10	13.25	14.17	0.0046	significant
Pure Error	4.67	5	0.9350			
Cor Total	5113.16	29				

The quadratic model was significant ($p < 0.0001$). The Lack of Fit was found to be significant ($F = 14.17$, $p = 0.0046$). This is due to the very low Pure Error variance ($MS = 0.9350$), observed because the six center-point replicates (Runs 25, 26, 27, 28, 29, 30) are tightly clustered with glucose yields ranging from 80.8% to 83.5%, making the Lack of Fit test very sensitive to minor deviations. Despite the significant Lack of Fit, the model's high R^2 (0.9732), adjusted R^2 (0.9481), and adequate precision (19.7342) collectively suggest reasonable predictive performance for describing the relationship between the process variables and glucose yield within the investigated domain [35]. The R^2 value of 0.9732 indicates that the model accounts for 97.32% of the variation in glucose yield. The adjusted R^2 (0.9481) and predicted R^2 (0.8494) were in good agreement, and the adequate precision (19.7342) demonstrated an adequate signal-to-noise ratio, suggesting the model could be used for designing experiments ().

The same analysis was performed for the experimental runs involving the Fe₃O₄@C-SO₃H catalyst, and the resulting quadratic model was used to describe the relationship between the investigated process variables and glucose yield.

Table 4. Fit Statistics for central composite design

Std. Dev.	3.02	R ²	0.9732
Mean	65.01	Adjusted R ²	0.9481
C.V. %	4.65	Predicted R ²	0.8494
		Adeq Precision	19.7342

The outcomes are shown in . As seen in a, pH had the greatest effect on glucose yield among all factors, with the yield increasing sharply from

37.2% at pH 3.0 to a maximum of approximately 82.1% at the optimal pH 5.0, before declining significantly to 56.4% at pH 7.0. This bell-shaped response likely reflects the combined influence of bulk pH, cellulose accessibility, and interfacial proton activity at the solid-acid surface, rather than a single dominant mechanism. The optimum-type behavior was also observed with the temperature profile (figure 7b): glucose yield increased from 42.6% at 30°C to nearly 82.1% at 50°C before decreasing to 67.8% at 70°C, likely reflecting the balance between enhanced molecular diffusion and reaction kinetics at moderate temperatures and possible limitations related to catalyst–substrate interactions or thermal effects at higher temperatures. For substrate loading (figure 7c), the highest glucose yield of approximately 82.1% was obtained at 3% (w/v) cellulose. The decline in yield at higher substrate concentrations may be associated with increased slurry viscosity and reduced effective contact between the cellulose substrate and the catalyst under the investigated conditions. Finally, the catalyst-loading profile (figure 7d) showed that glucose yield increased with increasing Fe₃O₄@C-SO₃H loading up to approximately 15 mg/g, beyond which further increases did not provide a proportional improvement in glucose yield. The lower response observed at 25 mg/g may indicate that increasing the catalyst amount beyond the optimum does not necessarily enhance substrate conversion under the investigated conditions, possibly because the available substrate becomes limiting relative to the catalyst amount.

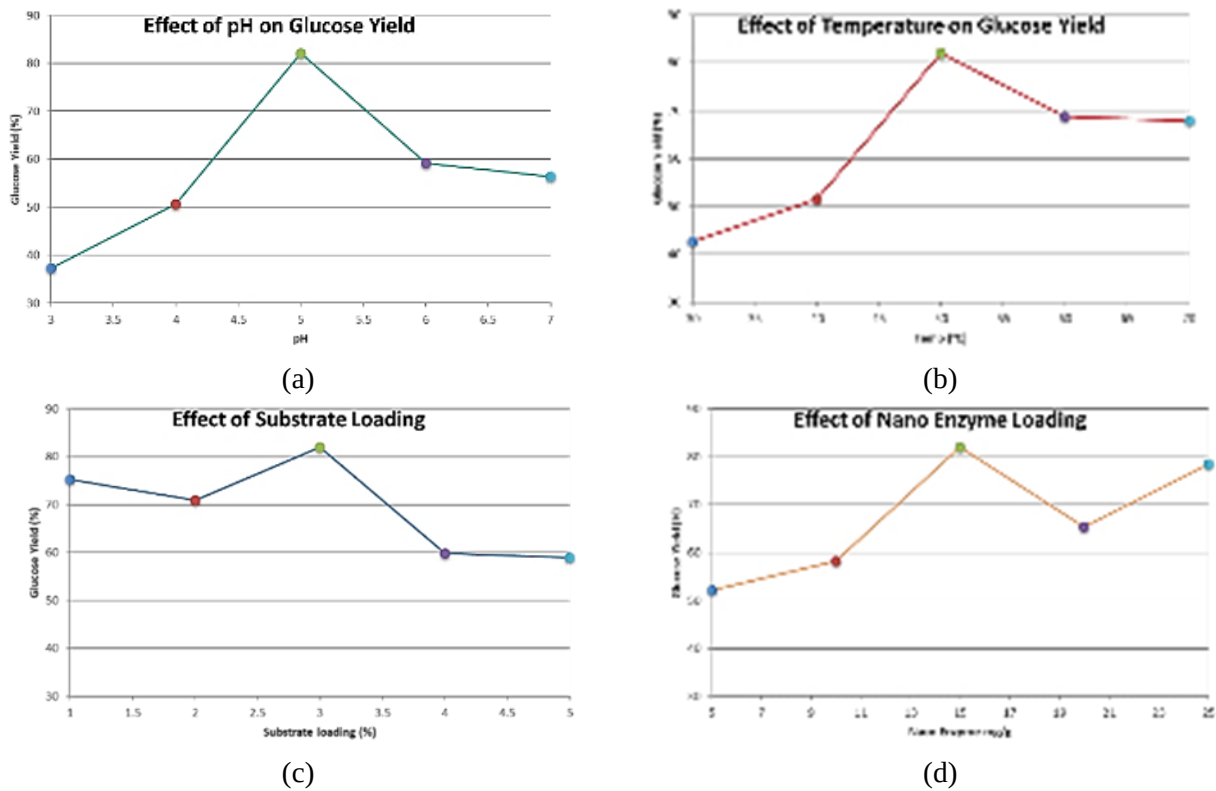
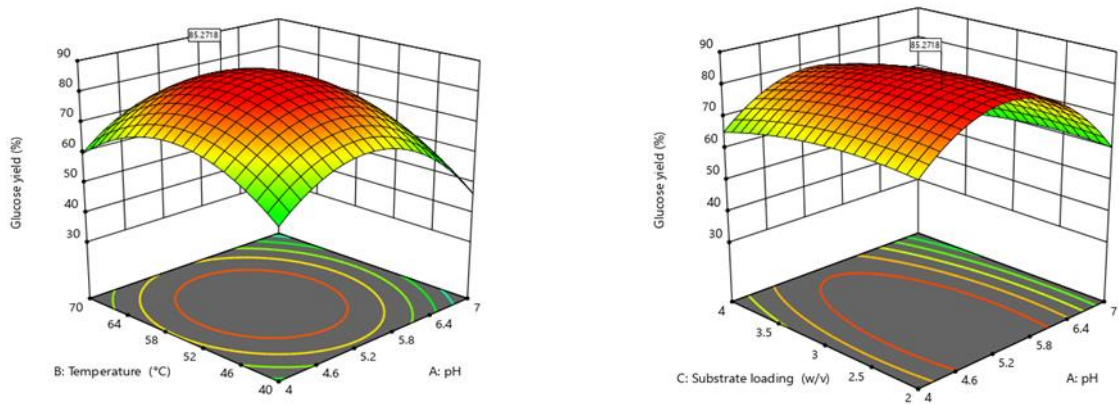


Figure 7. Effect of Each Factor on Glucose Yield (%)

3.5.2. Validation of the Experimental Model:
Model validation involved running the optimal predicted hydrolysis process parameters—using 2.5% substrate loading with 17.05 mg/g nano at pH

5 and 60°C. Model validation was performed by calculating the glucose yield (% w/w), which was 84.93%, close to the expected value of 85.27% ().



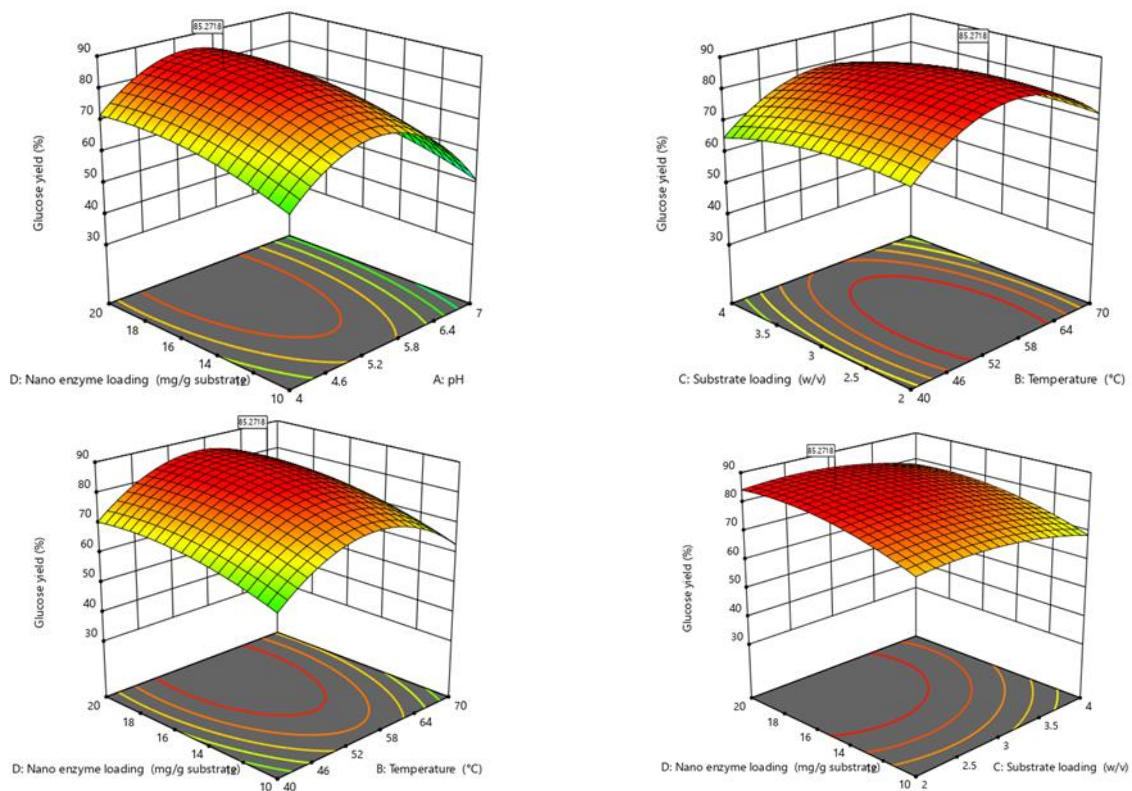


Figure 8. Model validation — predicted versus experimental glucose yield (%) for the Fe₃O₄@C-SO₃H solid acid catalyst

3.6. Reusability and Stability

Both Fe₃O₄-based systems demonstrated good recyclability for cellulose hydrolysis. In the case of cellulase immobilized on Fe₃O₄, most of the activity was retained after five cycles of hydrolysis (80%), a retention profile consistent with limited enzyme leaching and/or partial in-situ activity loss over repeated cycles, similar to reports of cellulase immobilized on iron oxide nanoparticles (figure 9). The magnetic solid acid catalyst Fe₃O₄@C-SO₃H also maintained high catalytic activity and magnetism after several cycles, consistent with other magnetic solid acid catalysts based on Fe₃O₄ for cellulose hydrolysis. The rapid recovery and reuse of both types of catalysts via magnetic separation suggest the feasibility of economical cellulose conversion. These reusability outcomes are competitive with or superior to recent literature. Hadadi and Habibi (2025) reported 66% activity retention after six cycles for cellulase on Fe₃O₄-RR120 [13], which is lower than the >80% retention after five cycles achieved in the present work. Gupta et al. (2025) observed 74–81% relative activity retention for individual cellulase components (CBH and EG) on NTA–Ni@Fe₃O₄ after five cycles, and the full enzyme cocktail retained approximately 78% after five cycles [36], comparable to the stability demonstrated here. For the solid acid system, Shekhanavar et al. (2024) demonstrated that Fe₃O₄@C-SO₃H synthesized

via a green route maintained stable magnetization and full catalytic activity for up to four consecutive recycling runs, with easy magnetic recovery under an external field [37], consistent with the stable magnetic recovery and catalytic performance observed for Fe₃O₄@C-SO₃H across multiple cycles in the present work.

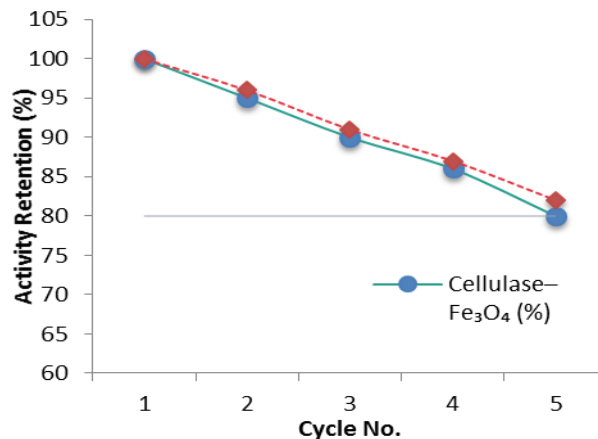


Figure 9. Catalyst Activity Retention Over 5 Reuse Cycles

Table 5. Reusability of Both Catalysts

Cycle	Cellulase–Fe ₃ O ₄ Activity (%)	Fe ₃ O ₄ @C-SO ₃ H Activity (%)
1	100	100
2	~95	~96
3	~90	~91
4	~86	~87
5	≥80	≥82

3.7. Comparison with Commercial Enzymes

The yields obtained with the optimized nanozyme were compared with those of commercial enzymes under their respective optimal conditions (). The $\text{Fe}_3\text{O}_4@\text{C-SO}_3\text{H}$ solid acid nanozyme produced an 84.93% glucose yield from cellulose, which is equivalent to 91.61% of the 92.7% yield achieved with commercial cellulases. These values ($84.93 \pm 0.01\%$ and $92.70 \pm 0.02\%$, technical triplicate) were statistically compared using Welch's two-tailed t-test, which indicated a significant difference ($t = 824.1$, $df = 2.6$, $p < 0.0001$); however, since these measurements represent technical replicates of the same reaction batch rather than independent process replicates (Section 2.8), this result reflects analytical measurement precision and should not be interpreted as evidence of a difference in the underlying process performance between the two catalytic systems.

While the Fe_3O_4 -based catalysts exhibited slightly lower sugar yields than commercial enzymes, they offered several advantages, such as activity across a broader pH and temperature range and recyclability. The Fe_3O_4 -based catalysts demonstrated hydrolytic activity over a wider pH range (4.0–6.5) and temperature range (40–65°C) than commercial enzymes (pH 4.5–5.5 and 45–55°C), potentially providing greater operational flexibility under variable process conditions. However, tolerance to common lignocellulosic inhibitors (e.g., furfural, HMF, phenolics) was not directly evaluated in this study and remains an important area for future research before assessing industrial applicability. It should be noted that the catalyst/enzyme loadings compared in this study are expressed on different bases: commercial enzymes were dosed based on activity (15 FPU/g cellulase, 30 CBU/g β -glucosidase), whereas the $\text{Fe}_3\text{O}_4@\text{C-SO}_3\text{H}$ solid acid catalyst and immobilized cellulase- Fe_3O_4 were dosed based on mass (mg/g substrate). Therefore, direct comparison of these loading metrics is not equivalent without normalization to a common activity unit. However, the commercial enzyme benchmark (92.7% yield) was obtained under strictly matched experimental conditions—same substrate batch, same reaction day, and identical GOD-POD analytical protocol—as the Fe_3O_4 -based catalyst systems, minimizing confounding batch-to-batch or measurement-related variability in the yield comparison. Establishing an FPU-equivalent activity basis for the Fe_3O_4 -based catalysts is a priority for future work to enable fully unit-normalized comparisons.

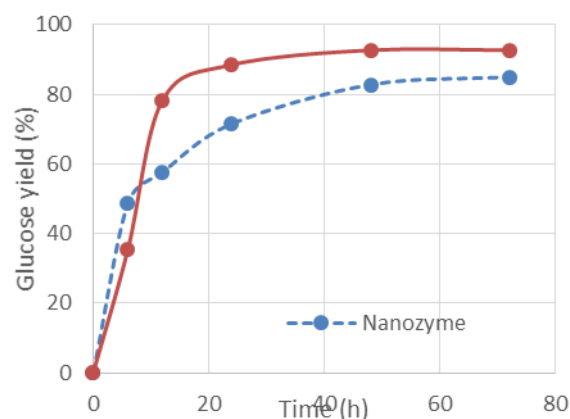


Figure 10. Reducing sugars produced by commercial enzymes (—) vs nanozyme (---).

This performance is significant when benchmarked against comparable systems in the literature. Xu et al. (2023) reported that cellulase immobilized on $\text{Fe}_3\text{O}_4@\text{ZIF-8}$ achieved a hydrolysis yield that was 20.44% higher than that of free cellulase at 15 FPU/g cellulose loading, while the immobilized system retained 71.03% of its original activity after four cycles [38]; however, the absolute glucose yields remain well below those achieved here. Gupta et al. (2025) demonstrated CMC-to-reducing sugar conversion using a three-enzyme NTA-Ni@ Fe_3O_4 cocktail with 78% relative conversion after five cycles [36], but this system required all three cellulase components simultaneously and involved complex purification steps, unlike the simple physical adsorption approach used here. The glucose yields ranged from 4% to 68% from cellulose under mild conditions [6], a range that the present $\text{Fe}_3\text{O}_4@\text{C-SO}_3\text{H}$ catalyst clearly surpasses at 84.93%. The 84.93% glucose yield obtained under RSM-optimized conditions (pH 5, 60°C, 17 mg/g catalyst, 2.5% substrate) represents a high conversion level under laboratory conditions and approaches the yield obtained with the commercial enzyme benchmark (91.6% of Celluclast 1.5L). The magnetic recoverability and reuse of the catalyst further support its potential for repeated applications. It should be noted that direct quantitative comparison across these studies is complicated by differences in substrate crystallinity and pretreatment history (Section 2.1.1); the present yields reflect hydrolysis of IL-pretreated, low-recalcitrance cellulose rather than untreated or highly crystalline standards used in some comparator studies.

Table 3. Comparison of glucose yields from cellulose hydrolysis using Fe₃O₄-based and related catalysts

Catalyst System	Glucose Yield (%)	Conditions	Reusability	Reference
Immobilized nanozyme	71.92	60°C, pH 5, atm.	-----	This study
Fe ₃ O ₄ @C-SO ₃ H (this study)	84.93	60°C, pH 5, atm. pressure, 12 h	>80% after 5 cycles	This study
Fe ₃ O ₄ @PS-SO ₃ H magnetic solid acid	64.14	140°C, 10 h, 1:1 acid/substrate	Good after 5 cycles	Liu et al. [32]
Carbon-based sulfonated solid acids (range)	4–68	Various mild conditions	Variable	Yang et al. [6]
Fe ₃ O ₄ @C-SO ₃ H green synthesis	Stable activity	Mild, 4 cycles	Full activity after 4 cycles	Shekhanavar et al. [37]
Cellulase on Fe ₃ O ₄ @ZIF-8	20.44% > free enzyme	50°C, pH 5, 72 h	71.03% after 10 cycles	Xu et al. [38]
Cellulase-RR120-Fe ₃ O ₄	Activity basis	37°C, pH 4.8	66% after 6 cycles	Hadadi & Habibi [13]
Cellulase cocktail on NTA-Ni@Fe ₃ O ₄	~78% relative conversion	Mild	~78% after 5 cycles	Gupta et al. [36]
Commercial Celluclast 1.5L	92.7	Standard conditions	Single-use	This work

Note: Reported yields across studies are not directly comparable due to differences in cellulose source, crystallinity, and pretreatment history, which strongly influence baseline substrate recalcitrance independent of catalyst activity.

4. Conclusions

Fe₃O₄ nanoparticles prepared via a simple coprecipitation method can be used as a multifunctional platform for developing immobilized cellulase biocatalysts and sulfonated magnetic solid acids for cellulose hydrolysis. The cellulase-Fe₃O₄ biocatalyst effectively hydrolyzes CMC under ambient conditions and can be recycled and reused with significant activity retention, overcoming several challenges associated with free enzymes. Magnetic Fe₃O₄@C-SO₃H solid acid also promotes cellulose hydrolysis, exhibits high acidity and magnetic responsiveness, and remains stable for recycling. These findings demonstrate the potential of low-cost, simple Fe₃O₄-based systems as magnetically separable catalysts for the sustainable valorization of cellulose. This study demonstrates the technical feasibility of applying two Fe₃O₄-based magnetic catalytic systems for cellulose

conversion at the laboratory scale. However, this work did not include a formal industrial-scale techno-economic analysis, which is a critical step before industrial implementation, including cost modeling and evaluation of complex real biomass process streams.

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