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Immobilization of cellulase on functionalized cobalt ferrite nanoparticles

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Abstract−Amine functionalized cobalt ferrite (AF-$CoFe_2O_4$) magnetic nanoparticles (MNPs) were used for immobilization of cellulase enzyme via 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDS) and *N*-hydroxysuccinimide (NHS) coupling reaction. The structural, morphological and magnetic properties of AF-$CoFe_2O_4$ were determined. TEM micrograph revealed a mean diameter of ~8 nm and showed that the AF-$CoFe_2O_4$ remain distinct with no significant change in size after binding with cellulase. Fourier transform infrared (FT-IR) spectroscopy confirmed the binding of cellulase to AF-$CoFe_2O_4$. The properties of immobilized cellulase were investigated by optimizing binding efficiency, pH, temperature and reusability. The results showed that the immobilized cellulase has higher thermal stability than free cellulase, which might be due to covalent interaction between cellulase and AF-$CoFe_2O_4$ surface. The immobilized cellulase also showed good reusability after recovery. Therefore, AF-$CoFe_2O_4$ MNPs can be considered as promising candidate for enzyme immobilization.

Keywords: Immobilization, Cellulase, Cobalt Ferrite, Bio-catalysis, Superparamagnetic Nanoparticles

INTRODUCTION

Bionanotechnology has now became one of the confidently recognized principle and focused sub-discipline within nanotechnology, in its own right [1]. Nano-sized magnetic particles have received increasing attention in various fields, including biomedical and environmental applications due to their size related properties, high specific surface area, and low toxicity [2-4]. Proper interaction is needed between nanomaterial and biomolecules for biomedical applications such as magnetic separation, drug delivery, enzyme immobilization, bacteria detection, hyperthermia therapy applications and protein purification [5-7]. For this, the exterior of MNPs needs to be suitably engineered to obtain improved colloidal stability in physiological media, biocompatibility and specific target ability [8-11]. Since there is diversity in biomolecules, it is always difficult to develop a surface modification method that carefully balances the intermolecular force between the biomolecule and the outer layer of MNPs [12]. Immobilization of bioactive molecules onto solid supports provides improved stability, reusability, modified catalytic properties, and in certain cases higher activity and selectivity [13]. The enzyme when immobilized on MNPs can be recovered easily by magnetic field. It is more feasible in large scale applications compared with other recovery methods such as filtration and centrifugation [14]. Regarding enzyme immobilization, it is important to analyze the surface interaction between magnetic particles and the protein molecules because of the role played by different surface energies. Binding of the protein (enzyme and antibody) is carried out by different technique such as surface adsorption, covalent interaction, hydrogen bonding [15,16]. Among these, covalent interactions are considered to be most suitable for industrial application because of improved reusability and stability [17].

The agro or municipal cellulose wastes are considered as an abundant renewable source which can be used as a substrate for the production of ethanol. This is an important source since it can be produced economically without using food resource. Cellulase is a key biocatalyst enzyme, which hydrolyzes the β-1,4glycosidic bonds of cellulose to produce glucose which further can be fermented for the production of bioethanol [18]. The practical applications of free cellulase are limited because of its hydrophilic nature [19]. For more diverse applications cellulase should be stable and reusable at a wide range of pH and temperature [20]. Therefore, an improvement in the stability and reusability is of great interest. Also, considering the high cost of cellulase and its contribution in the ethanol production by industry, development of proper immobilization technique is of particular interest [17]. Recently, Zang et al. reported chitosan coated Fe_3O_4 MNPs for immobilization of cellulase via glutaraldehyde activation [15]. Khoshnevisan et al. reported immobilization of cellulase on MNPs by physical adsorption [18]. But immobilization of enzyme through covalent interaction and proper surface functionalization technique is still a challenge. A more recent method of immobilization is use of carbodiimide cross linking chemistry by using 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDS) and *N*-hydroxysuccinimide (NHS), which allow an efficient conjugation with primary amine at physiological conditions.

The effectiveness of the immobilized enzyme varies with different functionalization and immobilization methods. In the present article, the cellulase enzyme was immobilized on amine functionalized Cobalt ferrite (AF-$CoFe_2O_4$) magnetic nanoparticles (MNPs) prepared by one-step method. Attempt was made to functionalize

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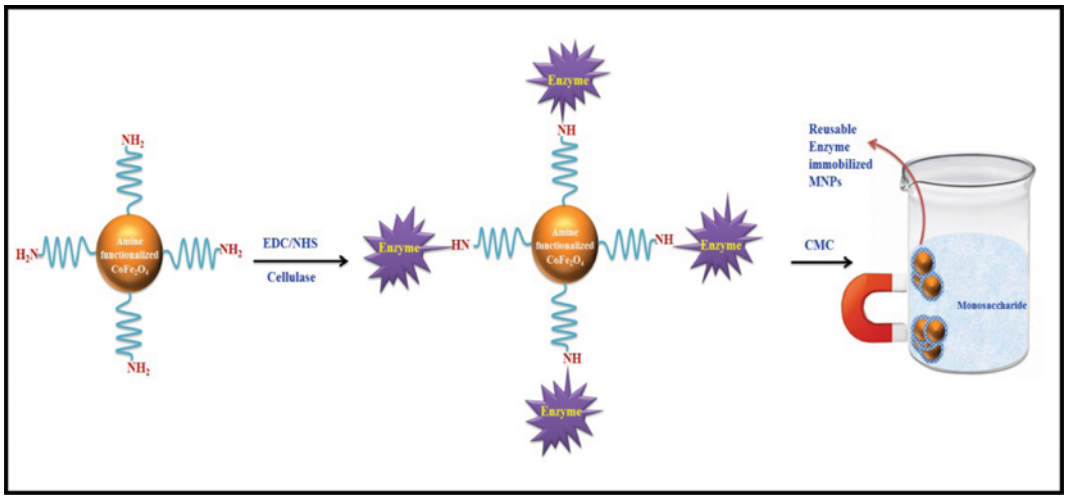


Scheme 1. The methodology of enzyme immobilization.

in single step, thus avoiding multistep functionalization methods generally used for immobilization process. As per best of our knowledge, most of the immobilization of enzyme has been reported on Fe_3O_4 MNPs but other ferrites are not explored in this direction. Cobalt ferrite ($CoFe_2O_4$) belongs to a family of AB_2O_4 type spinel ferrite (where A is divalent metal). It has been proposed for biomedical applications, since it is known to have large anisotropy compared to other oxide ferrites; also, magnetic properties and atomic properties can be controlled at atomic level through chemical modification. Furthermore, it shows excellent chemical stability, ease of synthesis, mechanical hardness, and electrical insulation. These mentioned properties make $CoFe_2O_4$ a promising candidate for bionanotechnological applications such as enzyme and protein immobilization [12,21]. The immobilization of cellulase on AF-$CoFe_2O_4$ is through carbodiimide activation. The size and structure of the resultant MNPs were characterized. Operating parameters for immobilized cellulase were evaluated using varying temperature, pH and reusability. In addition, possible binding mechanism of cellulase with AF-$CoFe_2O_4$ has been explained. Immobilization of cellulase can show a wide range of application.

EXPERIMENTAL

1. Materials

All chemicals used were of analytical grade and used without further purification. All metal precursors were taken from Molychem India. Diethylene glycol and ethanolamine were purchased from Thomas Baker. Bovine serum albumin, carboxy methyl cellulose (CMC), *N*-hydroxysuccinimide (NHS) and Cellulase from (*Trichodermareesei* ATCC 26921) ≥700 units/g were purchased from sigma-Aldrich. 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC*)* was purchased from spectrochem, India.

1-1. Apparatus

Reflux assembly for synthesis of AF-$CoFe_2O_4$, thermostat equipped magnetic stirrer, general glasswares and magnet for separation. The structural, morphological and magnetic properties of both AF-$CoFe_2O_4$ and cellulase immobilized AF-$CoFe_2O_4$ were studied by X-ray diffractometer (XRD), Fourier transform infrared spectroscopy (FT-IR) and transmission electron microscopy (TEM). Magnetization measurements were performed by using vibrating sample magnetometer (VSM). The methodology behind the work is presented in Scheme 1.

2. Synthesis of Amine Functionalized Cobalt Ferrite (AF-$CoFe_2O_4$) MNPs

Amine functionalized water dispersible MNPs were prepared by previously reported method with minor modification [12]. AF-$CoFe_2O_4$ MNPs were prepared by using a high thermal decomposition method. A stoichiometric amount of cobalt chloride, ferric chloride sodium acetate and diethylene glycol was heated continuously at 110 °C under stirring. Then 15 mL of ethanolamine was added and the reaction mixture was refluxed at 165 °C for 6 hr. The particles were washed several times with mixture of ethanol and ethyl acetate, separated magnetically and dried in a hot air oven.

3. Cellulase Immobilization

Fifty (50) mg of AF-$CoFe_2O_4$ was mixed with 5 mL solution containing EDC (0.093 mmol) and NHS (0.35 mmol). The resulting mixture was sonicated and refrigerated until the temperature reached 4 °C. Then cellulase enzyme solution was added to the above mixture followed with sonication. Finally, the cellulase immobilized AF-$CoFe_2O_4$ was recovered by placing the container on a strong permanent magnet.

4. Characterization

Phase identification and structural analysis of AF-$CoFe_2O_4$ and cellulase immobilized AF-$CoFe_2O_4$ were studied using XRD (Philip-3710) with Cr-K_α radiation (λ=1.5418 Å) in the 2θ range from 20° to 80°. The patterns were evaluated by X-pert high score software and compared with the Joint Committee on Powder Diffraction Standards (JCPDS) (card number-00-22-1086). The crystallite size was calculated from the full width at half maximum (FWHM) of the highest intensity diffraction peak, which is based on Debye-Scherrer equation,

$$t=0.9\lambda/\beta\cos\theta \quad (1)$$

where t is crystallite size, λ is the wavelength of Cu-K_α radiation, β

is FWHM and θ is the diffraction angle of strongest peak.

The morphology and size of the MNPs were determined from TEM micrographs. For this purpose, the colloidal solution of the MNPs was transferred onto a carbon-coated carbon grid and allowed to air dry. The grid was then scanned with a Philips CM 200 model TEM, with an operating voltage of 20-200 Kv and resolution of 2.4 Å. A Perkin-Elmer spectrometer (Model No-783 USA) was used to obtain FT-IR spectra of MNPs in the range of 450 to 4,000 cm^{-1} using KBr pellets, to confirm the amine functionalization and binding of cellulase on the surface of MNPs and to check the possible interaction between them. M-H curves were recorded to calculate the important magnetic properties like saturation magnetization and coercivity by VSM at room temperature. The binding of cellulase to AF-$CoFe_2O_4$ surface was determined with the Lowery method. The protein found in the removed supernatant was measured by a colorimetric method with bovine serum albumin as the standard. Glucose is measured by a glucose oxidase peroxidase method by using D-glucose as standard.

5. Cellulase Activity Assay

The enzymatic activity was determined by measuring glucose production after a reaction of cellulase immobilized AF-$CoFe_2O_4$ with cellulosic substrate. 0.5 mL solution cellulase immobilized AF-$CoFe_2O_4$ was added to 0.5 mL of cellulosic substrate (1% CMC) prepared in citrate buffer (pH 5). The resulting mixture was allowed to incubate at 50 °C for 30 minutes. After this, 0.5 mL of reaction mixture was withdrawn and glucose was measured. To minimize any interference, a series of blanks were measured in the same manner, one containing enzyme alone and another containing substrate alone. Enzymatic activity of cellulase was measured in the terms of amount of glucose produced (in µmol) per milligram of enzyme used over time, and is stated as IU/mg. The binding efficiency of cellulase to AF-$CoFe_2O_4$ was determined by evaluating the saturation of cellulase enzyme on the surface of AF-$CoFe_2O_4$. The effect of pH and temperature on cellulase activity was studied by using buffers of various pH ranging from 3-8 and incubation temperature of reaction mixture from 20-80 °C respectively.

6. Reusability Assay

Five (5) mg of cellulase immobilized on 50 mg of AF-$CoFe_2O_4$, was subjected to hydrolysis reaction having cellulose as a substrate, as mentioned earlier. After the specified reaction time the cellulase immobilized AF-$CoFe_2O_4$ was magnetically separated and introduced into fresh substrate and activity was measured. The procedure was repeated for six cycles.

RESULTS AND DISCUSSION

1. XRD Analysis

Fig. 1 shows XRD patterns of AF-$CoFe_2O_4$ and cellulase immobilized AF-$CoFe_2O_4$: phase formation and purity without any noticeable trace of impurities. The XRD pattern corresponds to the cubic spinel structure of AF-$CoFe_2O_4$ (JCPDS Card No. 00-22-1086). The Gaussian fit of the most intense peak (311) was used to calculate the FWHM for determination of the crystallite size (D) by Scherrer's formula. The average crystallite size calculated was ~6 nm, suggesting the formation of crystallites of nanosized. This observation confirmed that the amine functionalization and immobilization process did not lead to any impure crystal phase formation. In addition, the peaks appeared broad with a slight decrease in intensity in immobilized sample. This is due to the internal strain, which is induced due to decrease in signal-to-noise ratio owing to organic material present on the surface [22].

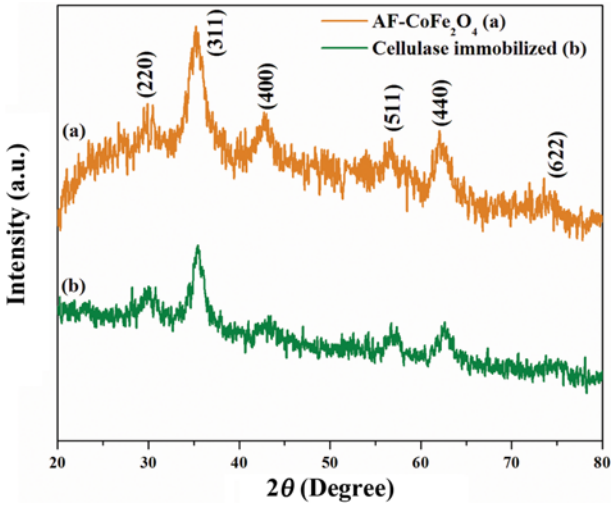


Fig. 1. XRD pattern of (a) AF-$CoFe_2O_4$ and (b) cellulase immobilized AF-$CoFe_2O_4$.

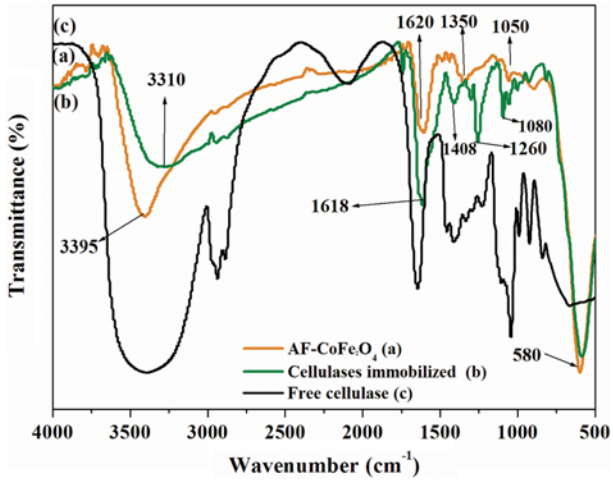


Fig. 2. FTIR spectra (a) AF-$CoFe_2O_4$, (b) cellulase immobilized AF-$CoFe_2O_4$, and (c) free cellulase.

2. FT- IR Studies

The FT-IR spectrum of AF-$CoFe_2O_4$, cellulase immobilized AF-$CoFe_2O_4$ and free cellulase is shown in Fig. 2(a), 2(b) and 2(c) respectively. The appearance of the peak at 580 cm^{-1} in Fig. 2(a) is attributed to Fe-O stretching vibrations, which is observed in all ferrites [22]. The peaks at 1,350, 1,620 and 3,395 cm^{-1} are due to C-N stretching, NH_2 scissoring and N-H stretching. This observation indicates the functionalization with amine on the nanoparticle surface. The obtained peaks all well match earlier reports [22,23]. The FT-IR spectrum of cellulase immobilized AF-$CoFe_2O_4$ is shown in Fig. 2(b). The presence of the peak at 580 cm^{-1} corresponds to metal-oxygen stretching in the ferrite lattice. The presence of characteris-

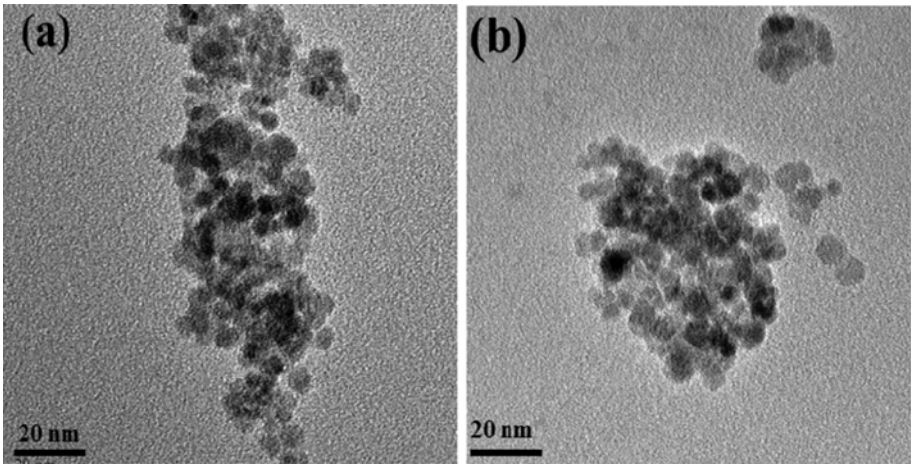


Fig. 3. TEM micrographs (a) AF-$CoFe_2O_4$ and (b) cellulase immobilized AF-$CoFe_2O_4$.

tic peaks at 1,618 and 1,408 cm^{-1} on the cellulase immobilized AF-$CoFe_2O_4$ confirms attachment of cellulase enzyme at the surface [24]. Also, shifting of peak is observed, which is due to amide bond formation, and this can be seen by the increased intensity of peak present at 1,618 cm^{-1}. Further, the double peak present at 1,260 and 1,305 cm^{-1} is due to the C-O stretching and the peak at 3,310 cm^{-1} is due -NH stretching. Thus, FTIR confirms the covalent attachment of cellulase enzyme on AF-$CoFe_2O_4$ surface.

3. Particle Morphology Analysis

Electron micrographs with size of AF-$CoFe_2O_4$ and cellulase immobilized AF-$CoFe_2O_4$ were obtained, as shown in Fig. 3. The MNPs are in the range of ~8 nm, which matches with crystallite size calculated from XRD pattern. It has been known that to support material small size provides large binding opportunities which are due to large surface area. TEM micrographs of AF-$CoFe_2O_4$ show slight agglomeration, which may due to the oriented growth mechanism that leads to the minimization of the surface energy, and it depends on the collective behavior of nanoparticles and intermolecular forces exist between them [25]. In the case of cellulase immobilized AF-$CoFe_2O_4$, the immobilization process did not cause any significant change in size, but a slight reduction in agglomeration has been seen when in Fig. 3, (a) is compared with (b).

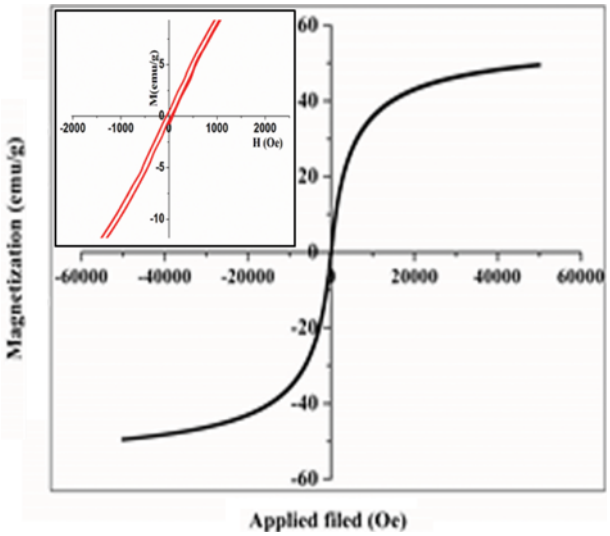


Fig. 4. M-H curve of AF-$CoFe_2O_4$. Inset show magnified view at origin.

4. Magnetic Studies

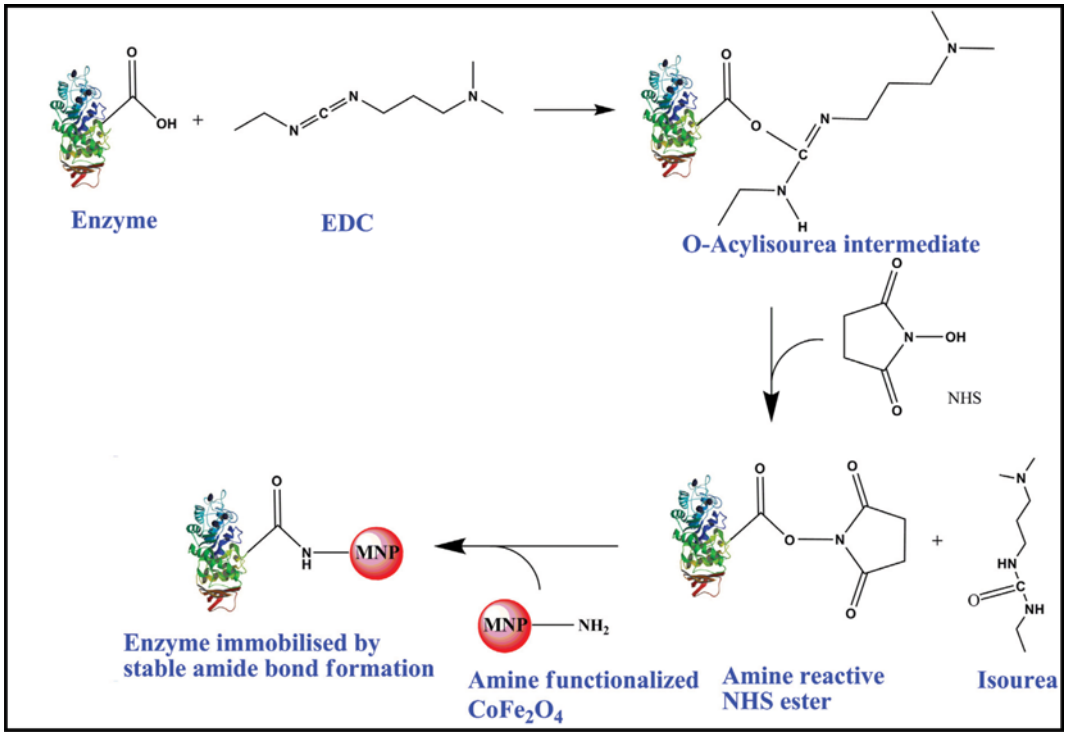
M-H curve of AF-$CoFe_2O_4$ at room temperature and applied filed of ±40 kOe at 300 K is shown in Fig. 4. The M_s value is 46.01 emu g^{-1}, which is smaller value as compared to the M_s value of bulk $CoFe_2O_4$, which is 80 emu g^{-1} [21]. This decrease in the M_s value could be mainly ascribed to the spin canting effect that becomes more dominant as the particles are in nano range [26]. Also, there is negligible coercivity and negligible remanence, which suggests the superparamagnetic nature of the sample at room temperature [27].

5. Mechanism of Cellulase Immobilization

Covalent linkage of cellulase over the surface of AF-$CoFe_2O_4$ was performed using EDC, which is an important member of the carbodiimide family and is water-soluble, ecofriendly, and non-cytotoxic [28]. The binding mechanism of the cellulase to AF-$CoFe_2O_4$ is shown in Scheme 2. EDC reacts with carboxylic acid groups of cellulase to form an active O-acylisourea intermediate, which is unstable in aqueous solutions and further reacts with NHS to create a stable (amine-reactive) intermediate, forming an NHS ester. It allows efficient conjugation to primary amines at physiological pH forming a stable amide bond linkage. Therefore, it can be concluded that application of N-hydroxysuccinimide (NHS) for enzyme immobilization on the carbodiimide activated MNPs enhance the efficiency of method [29]. To the best of our knowledge, no studies in this direction have been reported in the literature.

5-1. Optimization for Enzyme Binding

Here 50 mg MNPs were kept constant and enzyme loading was increased up to 25 mg. The amount of unbounded cellulase in the supernatant after immobilization was assayed. Optimum weight ratio of bound enzyme to MNPs was determined and optimum activity was measured. It has been found that at a concentration of 5 mg, there was maximum activity (Table 1). The relative activity curve for the varying weight ratio is shown in Fig. 5. At higher concentration, the percentage gets decreased and this may be due to the



Scheme 2. Covalent attachment of cellulase enzyme on AF-$CoFe_2O_4$ via carbodiimide cross-link chemistry.

Table 1. Relative activity value of immobilized cellulase and free cellulase

Cellulase added (mg)	Cellulase bound (mg)	Weight ratio (bound enzymes in mg/nanoparticles (mg)	Relative activity of immobilized cellulase (%)	Relative activity of free cellulase (%)
0	0	0	0	0
5	4.359	0.087	100	100
10	7.039	0.140	68.578	70.517
15	8.493	0.169	45.840	52.994
20	9.706	0.194	33.692	47.439
25	12.386	0.247	17.601	37.438

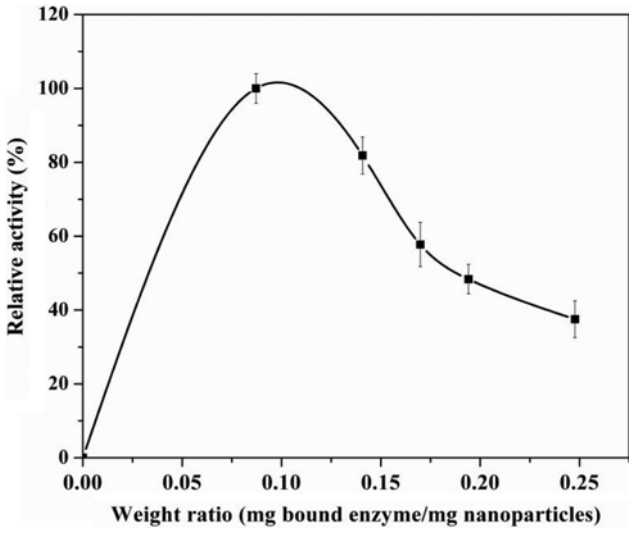


Fig. 5. Relative activity values corresponding to immobilized enzyme weight ratios.

surface area saturation and limited availability of binding sites [17].

5-2. Optimum pH and Temperature for Free and Immobilized Cellulase Enzyme

It is known that variation of solution pH strongly influences the ionic environment of an enzyme, thus affecting its interaction with the substrate and enzymatic activity. The effect of pH on the activity was investigated by carrying out an enzyme assay in the pH range of 3-8 at 50 °C, and the results are shown in Fig. 6. The maximum activity was at pH 5 for immobilized cellulase, which was the same for the free cellulase. From the result, there was a decline in enzyme activity, when pH condition was above 5.0. In alkaline pH media, the ionic groups within the cellulase molecule produce a strong electrostatic repulsion, leading to the stretching of the enzyme molecule, which results in the destruction and degeneration of the enzyme active center [30]. The immobilized cellulase showed a significant higher activity than the corresponding activity of free cellulase enzyme at the same pH condition. It simply indicates that immobilized cellulase possesses high pH stability. This may be due to covalent attachment of cellulase enzyme molecules with AF-$CoFe_2O_4$,

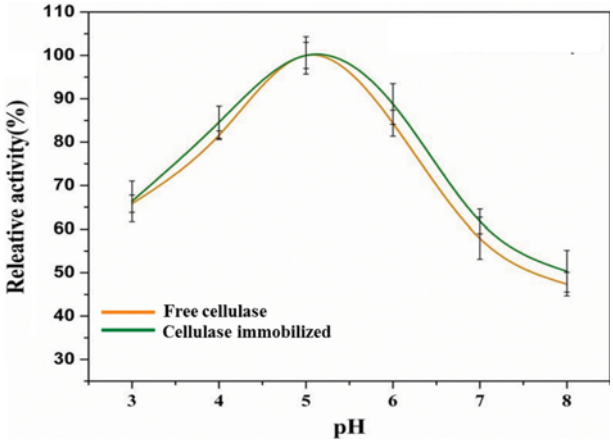


Fig. 6. Effect of pH on the activity of immobilized cellulase.

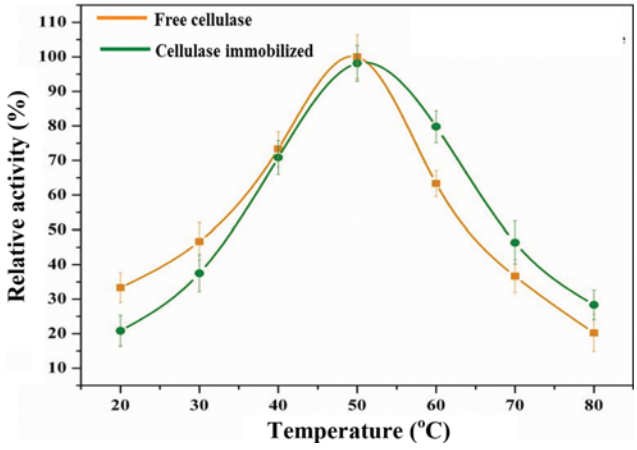


Fig. 7. Effect of temperature on the activity of immobilized cellulase.

which prevents the stretching of enzyme molecules. From above observation it is concluded that the immobilize cellulase shows better pH adaptability [31,32].

The effect of temperature on the activity was studied from 20 to 80 °C at pH 5. Fig. 7 indicates that 50 °C is the optimum temperature for immobilized and free cellulase. It has been seen that the free cellulase enzyme activity tends to decline as temperature increases above 50 °C, which implies that free enzyme should not be the first choice in industry as most of the reaction in the industry takes place at higher temperatures. In contrast, above 50 °C the activity of immobilized cellulase shows higher values compared to free cellulase. This is may be due to immobilization, which provides more firm external backbone for cellulase molecules. The effect of higher temperature in breaking of the interactions that are accountable for catalytic activity, and proper globular structure became less prominent, thus increasing the thermal stability of the immobilized cellulase [15,30,33]. This suggests that immobilization technique satisfactorily improves the thermal stability of the enzyme.

5-3. Recycling Efficiency of the Immobilized Enzyme

Since immobilization process enables the reusability of the enzymes for industrial applications, use of such immobilized enzyme can be repeated for catalytic activity after its magnetic recovery.

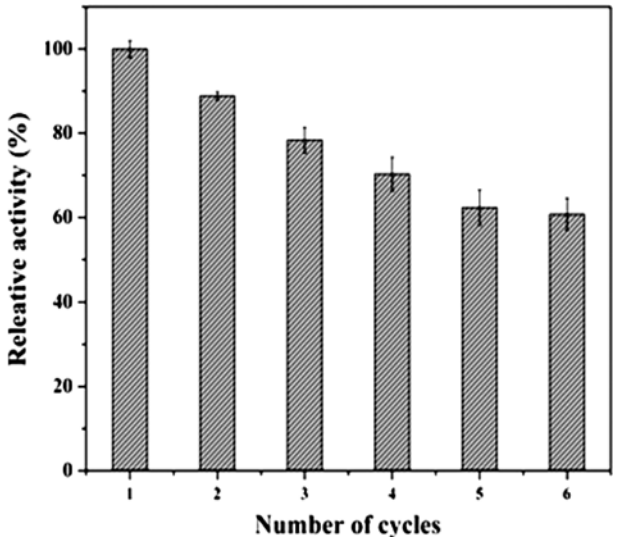


Fig. 8. Reusability of immobilized cellulase.

The results from this study are shown as a plot between the number of cycles and relative activity in Fig. 8. The activity of the immobilized cellulase is found to slowly reduce after the first cycle. Though the activity of the immobilized cellulase declined with the number of cycles, the initial activity was retained more than ~64% even after six cycles. This decrease in activity may be due to the slow denaturation of the enzyme during repeated handling [24] and also due to aggregation and loss of particles during magnetic separation. Another reason for decrease in activity is the removal of physically adsorbed cellulase which was present initially, but it might fall off during reusability assay [15]. The results obtained thus validate the recyclability of cellulase enzyme when immobilized on AF-$CoFe_2O_4$.

CONCLUSION

We have used AF-$CoFe_2O_4$ MNPs for cellulase immobilization, a process successfully carried out by using EDC-NHS coupling reaction. The higher magnetic sensitivity of immobilized cellulase (~46 emu g^{-1}) makes easy separation of the cellulase from reaction mixture. The optimum pH and temperature were 5 and 50 °C, respectively. The immobilized cellulase retains more than ~64% of the activity after six cycles. All the presented results suggest that AF-$CoFe_2O_4$ MNPs is a valid alternative for covalent immobilization of cellulase enzyme and may be considered for large scale industrial applications.

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