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Immobilization and characterization of cellulase on iron oxide nanoparticles for efficient re-usability

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Abstract

The high sensitivity to pH and temperature, as well as separation difficulties of free cellulase restrict efficient lignocellulose biotransformation. Enzyme immobilization on magnetic nanoparticles offers a new technique for stabilizing enzymes, with easy reuse. The study reports the synthesis of iron oxide magnetic nanoparticles (Fe_3O_4 MNps) for cellulase immobilization. By co-precipitating Fe^{2+} and Fe^{3+} in a 2:1 molar ratio, the Fe_3O_4 Nps was created. Cellulase was immobilized using glutaraldehyde as a cross linker. UV-Vis spectrophotometry, Fourier transform infrared (FTIR) Spectroscopy, Energy Dispersed X-ray spectroscopy and Scanning Electron Microscopy were studied to determine the surface plasmon resonance (SPR) band, functional group, element, size, shape and binding of the cellulase to the Nps. The impact of pH, temperature, stability and reusability of the immobilized enzyme were investigated. UV-peak at 320 nm indicates small nanoparticle size while FTIR bands at 500-700 cm indicate iron oxide Fe-O bonds vibrations. A characteristic peak at 1441 cm and 1435 cm for nanoparticles and immobilized cellulase indicates uncoordinated carbonate anion. Agglomeration of iron oxide nanoparticles (20 nm) was observed after cellulase immobilization. The optimum temperature of free cellulase shifted from 50 to 60°C after immobilization and the pH was stable at a range of 4 to 7. Better thermal and storage stability were displayed by the immobilized cellulase, which retained 71% activity after the fifth cycle of reuse. Immobilized cellulase-Fe_3O_4 MNps outperformed free cellulase in terms of pH tolerance, thermal stability, and storage stability. Additionally, because it can be recycled numerous times, it is commercially viable.

Keywords: Cellulase, Immobilization, Iron oxide Nanoparticles, Scanning Electron Microscopy, Energy Dispersion X-ray, Fourier Transform Infrared Spectroscopy, Re-usability

1. Introduction

Enzymes operate as catalysts in a variety of biological and chemical reactions. Cellulase is crucial because of its numerous applications in the textile, food, detergent, animal feed, and lignocellulose bioprocessing industries. Despite these significant advantages, the transformation of substrate into products utilizing cellulase faces a number of problems, including limited stability, high production costs, low recovery, and low reusability. [1, 2]. The aforementioned constraints are encountered when using free enzyme, rendering this strategy unsustainable. These constraints can be bypassed by immobilizing enzymes. Enzyme immobilization is the process of attaching an enzyme to the surface of a solid substance. Obtaining recyclable enzymes has a high industrial value because it reduces costs. In addition to reusability, effective immobilization techniques can improve enzyme overall performance by increasing enzyme activity, stability, purity, specificity, selectivity, and inhibitor resistance [3]. Enzyme immobilization ensures easy recovery, economic feasibility, and the capacity to endure changes in pH and temperature [4]. Immobilized enzymes are often more stable and easier to handle than unbound enzymes.

The immobilization of enzyme onto nanoparticles is of particular interest due to the reduced size of the nano-carrier, which improves the efficiency of the immobilized enzyme, and the smaller particle size of the carrier, which can provide a large surface area for binding, resulting in a higher surface area per unit mass particle [1]. Metal nanoparticles, particularly iron oxide nanoparticles, have a significant place among the numerous types of nanoparticles due to their superparamagnetic properties, tiny size, and wide variety of applications [5]. Iron oxide is biocompatible with the human system to some extent because the chelator of haemoglobin is composed of Fe (II) atoms. However, excessive concentration exposure can disrupt cells' regular physiological activity. This iron oxide can be conjugated with a variety of components to broaden its application. In industrial biotechnology applications, enzyme immobilized on nano particles is faced with problems in recovery e.g., via centrifugation and filtration [6]. However, magnetic nanoparticles (MNPs) have the benefit of being easily targeted and removed by the application of external magnetic fields; it also reduces the possibility of particle aggregation. [7]. Because of their distinct properties, MNPs are now used in conjunction with antibodies, nucleic acids, enzymes, and peptides. [8-10]. Gas-phase deposition, Pulsed laser ablation, Power ball milling, Co-precipitation, Micro-emulsions, Hydro-thermal synthesis, Sol-gel and electro spray synthesis are some of the physical and chemical methods used to create MNPs. Chemical co-precipitation is the most commonly utilized chemical technique [11]. Iron precursors are reduced to iron oxides using a weak reducing agent such as sodium hydroxide or ammonia in this process [12].

The current study aims to synthesize Fe_3O_4 MNPs via co-precipitation method and immobilize cellulase on the synthesized Nps. Further investigation was done on the characterization and reusability of the immobilized cellulase.

2. Materials and Methods

2.1 Materials

Cellulase from *Aspergillus niger* 22178-25G sigma-aldrich (activity 28 U/mL) was obtained from the Microbial Bioprocessing Laboratory, Department of Microbiology, University of Ilorin. Gluteraldehyde, $FeCl_3.6H_2O$, $FeSO_4.7H_2O$, Na_2HPO_4, NaH_2PO_4, carboxymethyl cellulose, 3, 5-di-nitrosalicylic acid were purchased from Hi media (India) and were of analytical grade.

2.2 Synthesis and activation of Iron Oxide Magnetic Nanoparticles (Fe_3O_4-MNPs)

The Fe_3O_4 nanoparticles were synthesized using the co-precipitation method described by Jiang [13] with some modifications. The co-precipitation involves Fe^{3+} and Fe^{2+} ions in NaOH solution under vigorous stirring and thermal conditions. In a nutshell, a 2:1 molar mixture of $FeCl_3.6H_2O$ and $FeSO_4.7H_2O$ was dissolved in water and carefully mixed. Chemical precipitation was accomplished by adding 0.9 M NaOH solution drop wise at 25 °C while vigorously stirring, the precipitates were washed three times with water and ethanol, then dried in an oven at 70 °C for four hours. The powder was stored at 4 °C for subsequent use. The production of iron oxide nanoparticles is indicated by a shift in color to black, and magnetism was demonstrated using a neodymium magnet placed beneath the glass container, with the nanoparticles being attracted toward the magnet. To activate the Fe_3O_4 MNPs, 5 g of MNPs were dissolved in ethanol and agitated for 10 min until completely disintegrated. A volume of 10 mL glutaraldehyde solution (10%) was added to the dispersed Fe_3O_4 and incubated for 2 hours at room temperature. To eliminate the excess glutaraldehyde, the particles were rinsed with deionized water.

2.3 Immobilization of cellulase on activated Fe_3O_4-MNPs

At 4 °C, activated Fe_3O_4 MNPs were kept overnight in a phosphate buffer solution of 0.1 M. By magnetic decantation, the activated Fe_3O_4 were collected, and the decanted Fe_3O_4 was then mixed with 50 mL of phosphate buffer (pH 6) containing 0.25 mL of cellulase with initial activity of 28 U/mL. The reaction was conducted for 24 hours at 30 °C with shaking at 100 rpm in an incubator shaker. The cellulase-bound Fe_3O_4 was then collected using a neodymium magnet and magnetic decantation before being thoroughly cleaned with deionized water [14]. The immobilized cellulase was stored at 4°C until further use.

2.4 Characterization of free and immobilized cellulase

Using a dual beam UV-Visible spectrophotometer (SPECORD 200 PLUS), absorbance spectra of solution for several wavelengths between 200 and 800 nm were taken to study the interaction of nanoparticles with UV-Visible light. The surface morphology of free and cellulase-immobilized nanoparticles was determined by scanning electron microscopy (SEM) (Joel JSM 6390) and the elemental composition was studied by energy dispersive spectroscopy analysis (EDS). The functional group characterization and binding of enzyme on nanoparticles were

evaluated by fourier transform infrared Spectroscopy (FT-IR) spectroscope (GX 2000, Perkin Elmer, USA) at 4000 to 400 cm wave number range.

2.5 Cellulase Assay

Based on the activity toward carboxymethyl cellulase (CMC), the cellulase activity was assessed. The reaction mixture included 0.5 mL of cellulase and 0.5 mL of 1% (w/v) CMC in 50 mM sodium phosphate buffer (pH 7). For 30 min, the reaction mixtures were incubated at 50 °C in a water bath [15]. The Miller [16] domain name system (DNS) method was used to measure the reducing sugar released. The CMCase activity, measured in units of enzyme activity per milliliter (U/mL), was defined as the quantity of enzyme needed to release 1 µmol of reducing sugars per minute under the specified assay conditions.

2.6 Effect of pH and temperature on CMCase activity and stability

The enzyme reaction was tested in a buffer with a pH range of 4-9. This was done to examine the impact of pH on the activity of free and immobilized cellulase. The free and the immobilized cellulase were inoculated in pH buffers (0.05 mM citrate buffer for pH range 3–5, 0.05 mM phosphate buffer for pH 6–9) and stirred for 30 minutes. Then samples were withdrawn, and their activities were determined using the DNS method (Described in section 2.5) By carrying out the process in a temperature range of 40 to 80 °C while maintaining phosphate buffer at pH 6.0 and incubation time at 30 min the effects of temperature on free and immobilized cellulase activities were also explored. By monitoring the enzyme's activity throughout 15 days of storage at 4 °C, it was possible to determine the storage stability of both free and immobilized cellulase. Relative activity was determined and was expressed as a percentage of the optimal activity at 100 %.

2.7 Reusability studies

The recyclability of the immobilized was investigated by repeatedly measuring the enzyme activity of the immobilized cellulase. The immobilized cellulase was added to 50 mL CMC solution (1% CMC in 50 mM phosphate buffer) and incubated for 6 h at 50 °C under shaking at 150 rpm. After each reaction activity measurement, the immobilized cellulase was separated with a magnet and washed several times with phosphate buffer solution before the next reaction and measurement was carried out. The residual activity was established after each cycle, with the activity considered to be 100% after the first cycle.

3. Results and Discussion

3.1 Characterization of Fe_3O_4-NPs-Cellulase

In the present study, the absorption spectroscopy of the synthesized magnetic nanoparticles was investigated at light wavelengths traversing from 300 to 800 nm (Figure 1). The UV-Vis spectrum of nanoparticles with a broad shifted surface plasmon resonance (SPR) band at 320 nm (peak region ranged between 310 and 350 nm). The peak band in this study is close to that of Kaur [17], who reported a peak band at 302 nm as an indication for the formation of smaller magnetic nanoparticles.

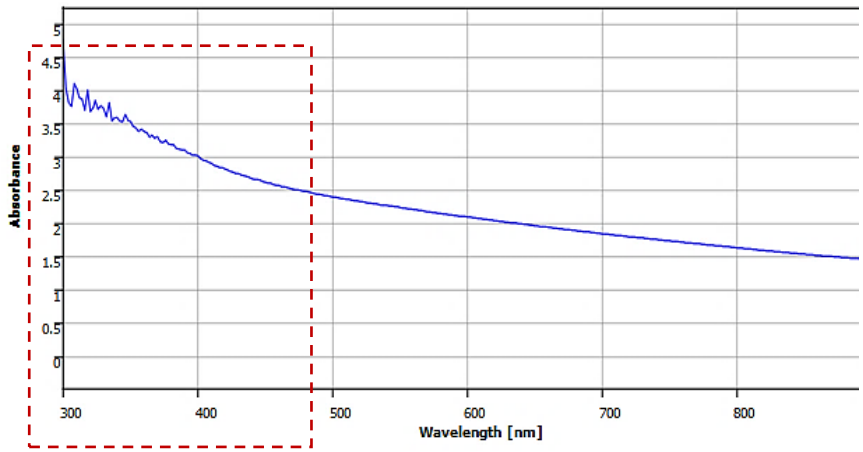

Figure 1 UV-Vis's spectrum of synthesized magnetic iron-nanoparticles.

The Fe3O4 Nps morphology and that of the immobilized cellulase were both visible in the SEM (Figure 2). The surface morphology of the Fe3O4 Nps showed a tightly packed arranged structure of sizes between 30-100 nm Following enzyme immobilization, rough surface structure particle aggregation with a large surface area comparable to those previously seen by Mohamed [18], were noticed on the NPs surface.

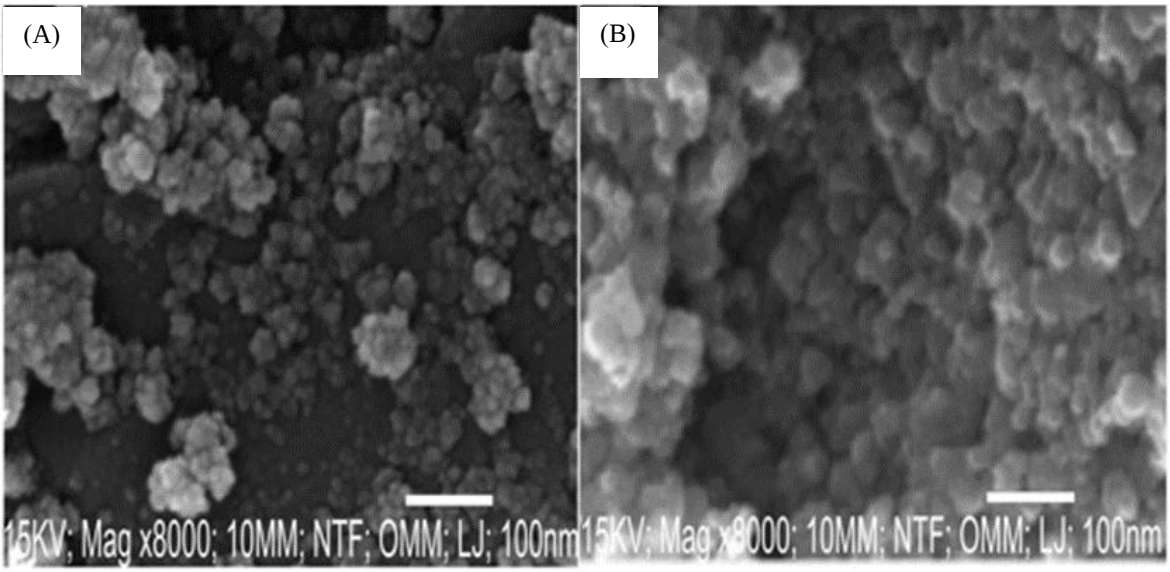

Figure 2 SEM images of (A) MNPs and (B) immobilized cellulase.

The binding of cellulase onto activated Fe_3O_4 nanoparticles was confirmed by Fourier transform infrared (FTIR) spectroscopy. The FT-IR spectrum of iron oxide nanoparticles showed a characteristic peak ranging from 3640 cm to 460.64 cm. The peaks at 3640 cm and 3445 cm showed the presence of O-H stretch of alcohol. The absorption peak at 2929 cm could be attributed to N-H stretch of amine salt. The intense absorption peak at 1612 cm showed the presence of C=C stretch of conjugated alkene. The sharp peak at 1441.45 cm corresponded to C-H bending alkane. The absorption peak at 1089.82 cm was identified as strong C-O stretching aliphatic ether. The bands at 500–700 cm were due to the vibrations of Fe–O bonds of iron oxide which confirmed that the synthesized nanoparticles were iron oxide nanoparticle Hwang [19]. In the spectrum of cellulase. the absorption peaks at 3785 cm 3703 cm and 3406 cm indicated the presence of O-H stretch of alcohol. The peaks at 2919 cm and 2855.82 cm have been identified as C-H stretching vibration of aldehyde and N-H stretching amine. The absorption peak at 2354 cm was attributed to the presence of O=C=O stretching Carbon dioxide. However, the 1601 cm peak in the cellulase enzyme indicated the overlapping amide band (due to protein backbone). Also, the absorption peak at 1442.53 cm could be C-H bending alkane. The presence of O-H bending of phenol and C-F stretch of fluoronated compound was shown by the peak at 1372 cm and 1280 cm. The absorption peak at 1038 cm could be attributed to asymmetry C-O-C stretch of aliphatic ester. The peak at 712.66 cm is attributed to C=C bend of alkenes. The hydroxyl, carboxylic groups, esters, aldehyde, and some amino acids may induce the production of cellulase. The observed characteristic peak of the immobilized cellulase ranged from 4189 cm to 366.66 cm. The peaks at 4189 cm, 3942.50 cm and 3883.70 cm showed the presence of medium sharp O-H stretch of alcohol while 3642.74 cm and 3446.33 cm absorption peaks could be attributed to broad O-H stretch of alcohol. The peaks at 2970.24 cm, 2930 cm and 2871.58 cm indicate the presence of NH^{2+} stretch of primary amide (bonded) and OH stretching vibration. The absorption peak at 2516.90 cm could be attributed to the presence of S-H stretch of thiol. The intense absorption peak at 1796.12 cm showed the presence of C=H stretch of aromatic compound. It is important to note that a distinctive peak for the uncoordinated carbonate anion that was seen in the Fe_3O_4-Nps spectra at 1441 cm relocated to 1435 cm in the immobilized cellulase. This characteristic shift in the frequency of cellulase bound Fe_3O_4-Nps from bare nanoparticles was due to the covalent binding of the cellulase enzyme onto the iron oxide nanoparticles [20].

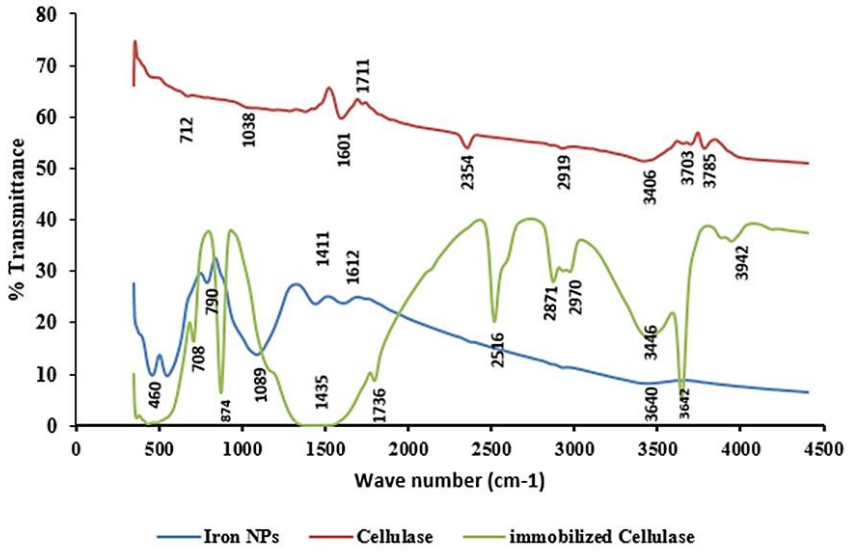

Figure 3 FT-IR spectra of Iron NPs, cellulase and immobilized cellulase.

EDX analysis revealed the intense peak of purely iron and oxygen which confirm the presence of the synthesized MNps (Figure 4). The oxygen and iron content of the naked magnetic Fe_3O_4 nanoparticles were reported to be 24.48 % and 75.52 % in weight respectively. Figure 4B revealed that immobilized cellulase, oxygen, carbon and iron content was found to be 18.34 %, 25.67 % and 60.96 %, respectively. The result is in agreement with Kouassi [21] which confirmed an increase in carbon from 3.2 to 3.74 % and oxygen from 28.67 to 34.75 % in nanoparticles after immobilization. The increase in both carbon and oxygen content of cellulase-immobilized nanoparticles was as a result of the binding of cellulase enzyme onto the surface of nanoparticles a [19].

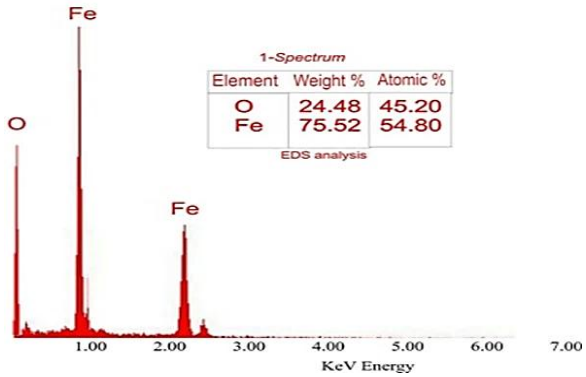

Figure 4 (**A**) Energy dispersive X-ray analysis of free magnetic iron-nanoparticles.

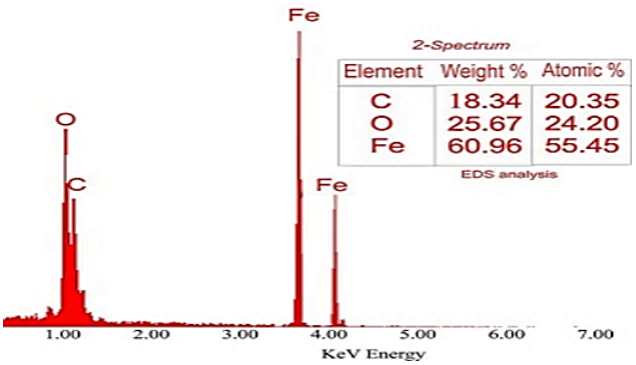

Figure 4 (B) Energy dispersive X-ray analysis of immobilized cellulase.

3.2 Effect of pH, temperature, storage stability and reusability of free and immobilized cellulase

It was observed that the free enzyme exhibited maximum activity at pH 5.0 while the immobilized cellulase showed maximum activity at range pH 5.0 -7.0 (Figure 5). The activity of the free and immobilized cellulase was set at 12 U/mL for these studies. It is interesting to note that the cellulase displayed a characteristically broad pH when bonded to Fe_3O_4-Nps. The tight interaction formed between the nanoparticle and the cellulase permits the immobilized cellulase to be more stable at both acidic and alkaline pH than the free enzyme. At alkaline pH the free cellulase showed decreased activity compared to the immobilized cellulase. This phenomenon was explained by Selvam [6] where the alkaline medium reduced free enzyme activity due to electrostatic state while nanoparticles enhance cellulase-amino bond resistance in high-alkalinity mediums. At pH 8, the immobilized cellulase was able to retain 75 % of its activity when compared to 41.43 % relative activity displayed by the free cellulase. Also, the higher resistance of immobilized cellulase to the pH alterations is related to the reduction of their mobility as well as retention of the organized structure and alteration after immobilization. Previous researchers have also reported a broader pH stability of immobilized cellulase compared to the free cellulase [17].

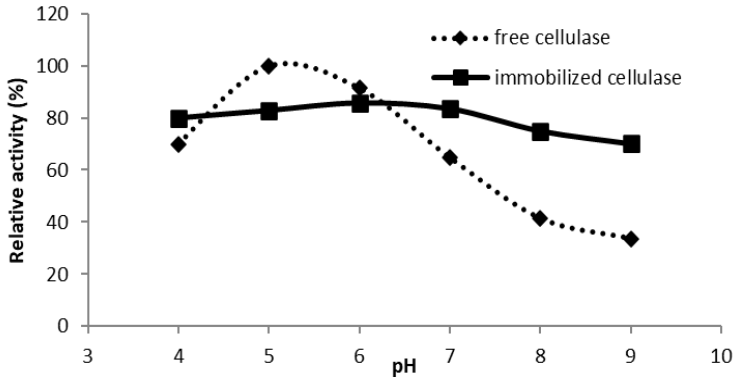

Figure 5 Effect of pH on activity of free cellulase and immobilized cellulose.

The optimum temperature for the free cellulase shifted from 50 °C to 60°C upon immobilization (Figure 6). Immobilized enzymes' temperature optima may increase due to decreased flexibility and increased tolerance to unfolding and denaturation due to conformational changes [22]. The immobilized cellulase showed higher activity at higher temperatures (60 °C to 90 °C) than the free cellulase. Remarkably, 80.1 % of the activities the immobilized cellulase was retained at 70°C while the activity of the free cellulose reduced drastically to 66 %. However, at 90°C, the activity of the immobilized cellulase was still maintained above 50 %. Immobilized enzyme displayed 59.3% of its activity at 60°C, but free enzyme only preserved 26.5%, according to Ren [14]. This demonstrated that the cellulase immobilized on iron oxide nanoparticles displayed higher heat tolerance which could be as a result of restriction of the thermal mobility. Another theory is that the nanoparticle surrounds the cellulase and creates a barrier that prevents the transfer of heat to the cellulase, shielding it from damage. The optimum temperature for the free cellulase shifted from 50 °C to 60°C upon immobilization. The results were consistent with previous studies where immobilized cellulase displayed optimal temperature at 60 °C as compared to free cellulase with optimal activity at 50 °C [17]. Cellulase immobilized on iron oxide nanoparticles demonstrated a shift in optimal activity from 50 °C to 55 °C in a study by Selvam et al., (2016). However, according to Sillu and Agnihotri [23], the free and immobilized cellulase on magnetic halloysite nanotubes-maintained temperature optimal at 50 °C.

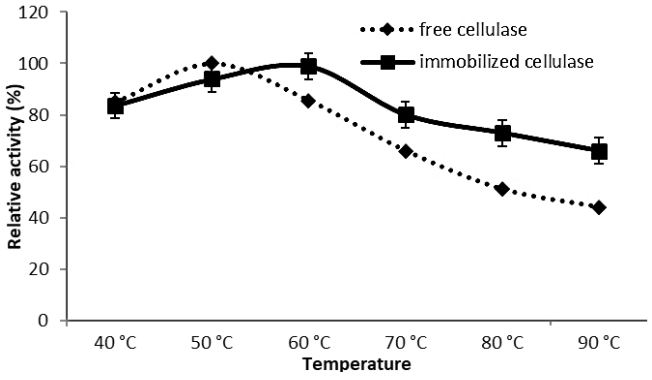

Figure 6 Effect of temperature on activity of free cellulase and immobilized cellulase.

Improvement in thermal stability properties could be influenced by immobilization of enzyme. The enzyme was incubated at 60°C for 1-5 h for the thermostability studies (Figure 7). According to our findings after being incubated for 2, 4, and 5 h, free cellulase preserved 71%, 55%, and 22% of its activity. In the meantime, the immobilized cellulase maintained 82%, 66%, and 35% of its activity, respectively, under the same circumstances. This implies that the activity of immobilized cellulase declines more slowly than that of free cellulase. This observation could be attributed to the enzyme's decreased movement, rearrangement of structure, and higher molecular rigor. These can lead to an improvement in the enzyme stability [24]. The result is consistent with the previous study where free cellulase lost 47.4 % of its activity after incubation at 60 C for 2 h while immobilized cellulase still retained about 73.4 % activity in a similar condition [17]. Similarly, incubation of free cellulase at 70 °C for 6 h severely affected the enzyme and reduced the residual activity to about 12.3 % while about 76.6 % activity of the immobilized cellulase was maintained at the same condition [23].

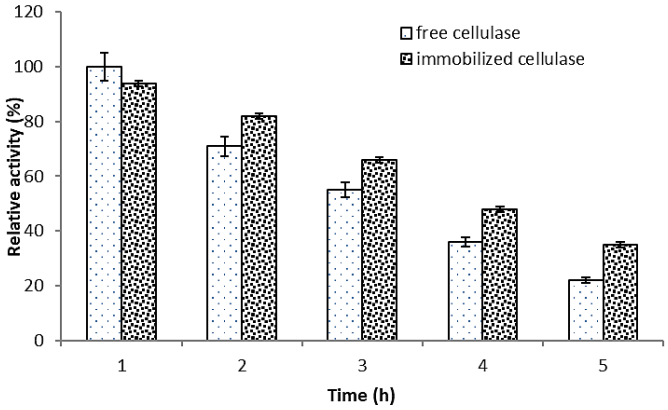

Figure 7 Thermal stability of the free and immobilized cellulase at 50 ºCover 5 hours of incubation.

Considering the fact that enzymes are affected by environmental conditions. It will be very interesting to examine their storage stability at room temperature. The relative activity of the free and immobilized cellulase declined to 92% and 98.2%, respectively, after three days of storage at room temperature (Figure 8). After 15 days, the relative activities of the immobilized cellulase were maintained at 79 % while that of free cellulase dropped to 58%. It is worth nothing that the activity of the free cellulase declined sharply after day 6 of storage till the day 15. However, that of the immobilized cellulase depict a linear declination. Similar linear and sharp declination was previously observed [23]. Fastening cellulase to iron oxide nanoparticles enhances elasticity and toughness, making it more durable and able to withstand harsh environmental conditions. Similarly, it could be inferred that attaching cellulase to iron oxide nanoparticles allows for flexibility and toughness, allowing it to survive harsh environmental conditions, making it more robust. Better storage stability has been recently reported for enzymes on magnetic support [25-28].

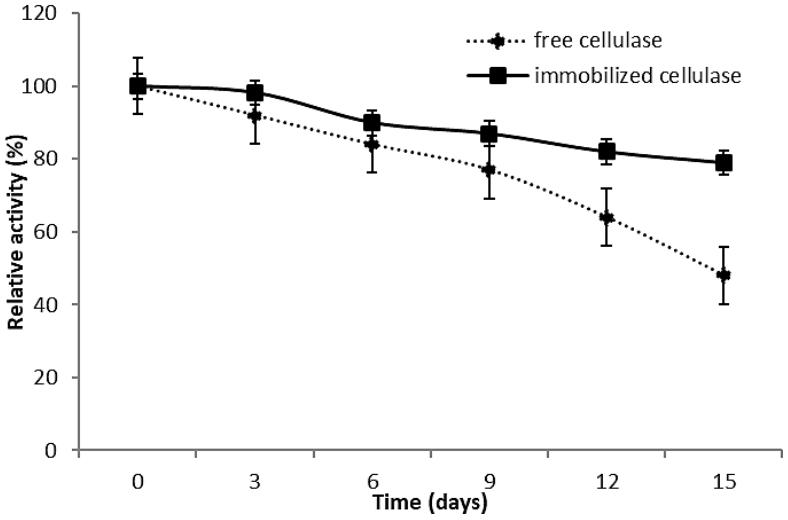

Figure 8 Storage capacity of immobilized cellulase and free cellulase over 15 days.

Reusing enzymes in industrial processes has positive economic benefits. Although the activity of the immobilized cellulase dropped after each cycle, it was still able to maintain 68% of its maximum activity after 6 cycles of reuse of cellulase immobilized Fe_3O_4-Nps (Figure 9). The drop-in activity can be a result of recurrent wear, separation, or enzyme leakage from the enzyme-nanoparticle complex as a result of poorer bonding. Recyclability studies have shown different variation in previous studies. According to the Jia et al [29], only 38% of activity was remained after 4 cycles of reuse of cellulase immobilized on iron oxide nanoparticles. However, cellulase immobilized on chitosan-coated magnetic nanoparticles maintained 80% of its activity after 15 cycles of reuse [30].

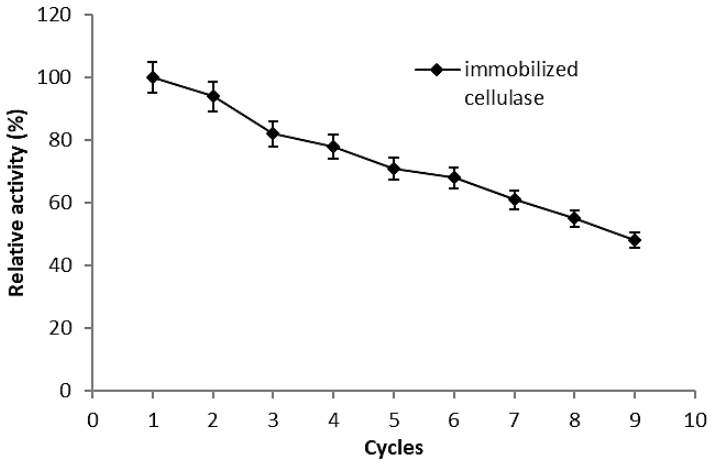

Figure 9 Reusability of the immobilized cellulase.

Meanwhile, according to Muley [31] after 5 cycles of application of cellulase immobilized iron oxide nanoparticles, 81.15% of the initial activity was maintained. According to Sillu and Agnihotri [23], lower residual activity as a result of recyclability could also be linked with inactivation mechanism. The magnetic nature of the nanoparticles facilitated easy recovery of the immobilized cellulase for reuse.

4. Conclusions

Cellulase was immobilized on Fe_3O_4 magnetic nanoparticles in this study using the co-precipitation technique. The FTIR analysis display high interaction between the cellulase and the Fe_3O_4MNPs. The surface morphology and characteristics of the immobilized cellulase was verified by the SEM and EDX analysis. Immobilized cellulase confers more resistance to temperature, acidic/alkali pH and storage stability as compared to the free cellulase with magnetic recovery, the immobilized cellulase also confers superior reusability. The study suggested that the immobilized cellulase capabilities could be beneficial for industrial use.

5. Conflict of Interest

Authors declared they have no conflict of interest.

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