Cellulase immobilization on TEOS encapsulated and APTES functionalized magnetic nanoparticles (MNPs)

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Abstract. Immobilized enzymes are more stable and more resistant to environmental changes in contrast to free enzymes in solution. Magnetic nanoparticles (MNPs) are potential immobilization supports materials for enzymes and easily recovered for reuse. It has low toxicity effects and fast separation from reaction mixtures simply by use of external magnets. Enzyme immobilization technology also helps to boost the enzyme's properties by using the technique to keep it in separate support or matrix. The aim of immobilization is to render the enzyme immobile so that it is reusable during the reaction, which leads to a more efficient process and easy separation from the substance. The aims of this investigation were to encapsulate (coat) and functionalize MNPs by using TEOS and APTES respectively for reusable cellulase immobilization on that as a supports matrix. The silicon encapsulation with TEOS and amino functionalization of synthesized MNPs was confirmed by the FT-IR and XRD. Cellulase was immobilized successfully on that nanoparticles and also confirmed by FT-IR, cellulase assay. The immobilized enzymes showed a satisfactory level of reusability (51%) after 10 consecutive reaction cycles.

Key words: Magnetic nanoparticles (MNPs; encapsulation; functionalization; immobilisation; cellulase; reusability.

1. Introduction
Enzymes are biological catalyst that catalyzes many chemical and biochemical reactions universally present in microbes, plants and animals. Enzymes have extensive applications in food, textiles, paper & pulp, detergents, health care & pharmaceuticals and chemicals industries. Owing to their lack of long-term operating reliability, time cycle, and recovery & reusability, attractive characteristics of enzymes and their widespread industrial applications are often obstructed. One of the methods for reducing these problems is enzyme immobilization [1]. Immobilized enzymes are more stable and more resistant to environmental changes in contrast to free enzymes in solution. More significantly, the heterogeneity of immobilised enzyme systems allows for the fast recovery of both enzymes and
products, the multiple reuse of enzymes, the continuous functioning of enzyme processes, the rapid termination of reactions, and a wider range of bioreactor models [2].

The use of immobilised enzymes is now a common practice in the pharmaceutical, chemical and food industries to produce many finished products [3]. Enzyme immobilisation technology also helps to boost the enzyme's properties by using the technique to keep it in a separate support or matrix. The aim of immobilisation is to render the enzyme immobile so that it is reusable during the reaction, which leads to a more efficient process and easy separation from the substance. Finally, the thermal stability of the enzyme and its resistance to degradation, denaturation and aggregation are also improved by immobilization [4]. In determining the performance of the immobilized system of enzymes, the characteristics of the matrix are important. Typical support properties include physical compression resistance, hydrophilicity, enzyme inertness, ease of derivatization, biocompatibility, microbial attack resistance and low-cost availability [5]. For enzyme immobilisation, a number of inorganic supports are often used, e.g., alumina, silica, zeolites, and mesoporous silica. Silica-based substrates are the most suitable matrices for the immobilisation of enzymes in the industrial manufacture and study of enzyme-processed products. Carriers with a wide surface area still make a great contribution to achieving good efficiency in immobilization [6].

Magnetic nanoparticles (MNPs) are potential supports matrix and are readily retrieved for reuse for bioactive materials such as peptides, enzymes, antibodies, and nucleic acids. Their distinct advantage is their simple isolation from reaction mixtures by the use of external magnets, which have general low toxicity. Researchers have clearly demonstrated improved lipase enzyme function, loading and stability using MNP immobilised enzyme [7]. The aims of this investigation was to coat (encapsulate) and functionalize of MNPs by using TEOS and APTES respectively for reusable cellulase immobilization on that as a magnetic nanoparticles.

2. Methodology, Results and Discussion

Cellulase from Aspergillus niger was collected from commercial sources (Sigma) and the supporting chemicals like tetra-ethyl orthosilicate (TEOS), 3-amino propyl tri-ethoxy silane (APTES), glutaraldehyde, bovine serum albumin (BSA), dinotrosalisaylic acid (DNS), carboxymethyl cellulase (CMC), FeCl₃, KI. The synthesized MNP, encapsulated, functionalized and immobilized nanoparticles was characterized by using Transmission electron microscopy (TEM; Brand: Fei; Model: Tenai-G2-20-Twin), Vibrating sample magnetometer (VSM), Fourier transform infrared (FTIR; Perkin Elmer FTIR-ATIR 100 spectrometer), X-ray diffractometer (XRD). The details methodology and results is discussed in following subsections.

2.1. Synthesis of MNPs, Encapsulation, Functionalization and Activation

Magnetic nanoparticles (MNPs) was synthesized by following the method described by Khalil [8] with modification of temperature, time and drying system. Molar ratio of FeCl₃ and KI potassium iodide will be 3:1 (the data is not submitted here). Encapsulation (silica coating) with TEOS of synthesized MNPs was performed the method described by Fortes, Daniel-da-Silva, et al, [9] with modification. Silica were deposited onto the core shell of MNPs and surrounded over it. Briefly, prior to silica encapsulation 100 mg of MNPs was dispersed in a conical flask with ethanol-water solution (3:1) by 30 min sonication to prevent agglomeration. After that 0.1 ml 98% TEOS and 0.1 ml 25% ammonia solution was added drop-wise then the mixture was shaken for 24 h. The TEOS coated MNPs was decanted with external magnet, washed several times with DI water and dried at room temperature. Subsequently, the surface of silica coated nano particles was functionalized with 3-aminopropyltriethoxysilane (APTES) by slight modification of Ladole et al, method [10], [11]. Functionalization of MNPs made more attachments sites (chains) for coupling agents like glutaraldehyde. Briefly, the TEOS coated dried MNPs was dispersed again in a conical flask with ethanol-water solution (3:1) by 30 min with sonication and adding drop-wise of 0.1 ml of 97% APTES solution. Then the mixture was shaken again for 16 h. After that the MNPs was separated by external magnet and washing several times with DI water and dried at room temperature. The functionalized
MNPs was activated by 0.5 ml 25 % glutaraldehyde under continuous shaking at room temperature for 3 hours in acetate buffer (pH; 5.0) for cellulase immobilization. Here, activation of MNPs with glutaraldehyde was facilitated the nano supports to anchor the enzymes (cellulase) molecules. After several washings with the same buffer this activated MNPs will be used as supporting materials for enzyme immobilization.

2.2 Cellulase immobilization, activity determination and reusability performance

A reaction volume of 10 ml acetate buffer pH 5.0 containing 1.0 mg/ml cellulase powder concentration was used in each experiment with triplicate and the resulting solution was shaken for 3 hours at 130 rpm in rotary shaker at 30°C. Then the enzyme immobilized nanoparticles was removed by magnet from the reaction mixture (solution) and washed. Bradford method was used to measure the quantity of protein in the supernatant [12]. By subtracting the protein recovered in the supernatant from the protein subjected to immobilization, the immobilized enzymes sum was determined. The immobilization percentage (equation 1) was determined from the difference between the amount of protein bound to MNPs and the amount of protein present in the free enzymes solution. [10].

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\text{Percentage of immobilization} = \frac{C_i V_i - C_o V_o}{C_i V_i} \times 100
\]

(1)

Where, \(C_i\) = initial protein content (mg/ml), \(V_i\) = initial volume of reaction mixture (ml). \(C_o\) = final protein content (mg/ml), \(V_o\) = final volume of reaction mixture (ml).

Cellulase activity in soluble as well as immobilized was determined by the standard application of the colorimetric DNS method [13] and modification of Kumari et al, method [14] which implies, one unit of activity as the amount of enzyme required to produce 1 μmol of reducing sugar/min. CMC solution was used as substrate. This method is based on quantification of colour development as a function of time after reaction between DNS and the reducing sugars liberated through enzyme activity.

2.3 Characterization

The synthesized MNP, encapsulated, functionalized and immobilized nanoparticles was characterized by using Transmission electron microscopy (TEM; Brand: Fei; Model: Tcnai-G2-Twin), Vibrating sample magnetometer (VSM), Fourier transform infrared (FTIR; Perkin Elmer FTIR-ATIR 100 spectrometer), X-ray diffractometer (XRD).

2.4 Results and Discussion

The size and magnetization of synthesized magnetic nanoparticles was determined by TEM and VSM (the data and image is not submitted here). The transmittance peak at 558 to 592 cm\(^{-1}\), 796 cm\(^{-1}\) of FTIR spectrum (figure 1) confirms the presence of the functional group Fe-O, Si-O-Fe bond exists within the encapsulated and functionalized MNPs that is indicated the SiO\(_2\) anchored on Fe\(_3\)O\(_4\) (magnetic nanoparticles). The strong band at 1085 cm\(^{-1}\), 1477 cm\(^{-1}\) denotes the presence of Si-O-Si, NH of APTES respectively which indicated the presence of free NH\(_2\) groups on the MNPs surface. This identification of encapsulated and functionalized MNPs is supported by Fortes, Daniel-da-Silva, et al, [9]. The FTIR spectrum of the cellulase immobilized MNPs was shown on figure 2 which showed some characteristics peak at 1551 cm\(^{-1}\)(-CONH amide bonds) 1630 cm\(^{-1}\)(-CONH amide bonds) 3447 cm\(^{-1}\) is attributed to the stretching of N-H from free NH\(_2\) group along with the presence of Si-O and Fe-O peak was observed 962 cm\(^{-1}\) and 596 cm\(^{-1}\) respectively. This findings of revealed anchoring of cellulase onto the functionalized MNPs [15].
The X-Ray diffraction (XRD) patterns of the dried black powder was obtained within a 2θ range from 10 to 80° by using XRD (Rigaku Mini FLEXII) with CuKa radiation at the operating voltage of 2.7 kW. From the XRD data the phase and purity of iron oxide black powder was determined by comparing the JCPDS code. XRD analysis of TEOS coated MNPs showed that similar pattern as that of bared MNPs (figure 3) the sharp diffraction peaks in both XRD pattern clearly confirm that magnetic product is well defined crystallite, without any phase change and impurity diffraction peaks [15].
Figure 3. XRD of TEOS coated (encapsulated) magnetic nanoparticles (MNPs) and bared MNPs.

Cellulase immobilization efficiency was 96.75% and reusability of immobilization cellulase on MNPs was 51% after 10 consecutive reaction cycles (figure 4).

Figure 4 Reusability of immobilized cellulase on MNPs.

Conclusion
Cellulase was successfully immobilized on TEOS coated (encapsulated) and APTES functionalized magnetic nanoparticles (MNPs) which easily separable by external magnetic force from the reaction mixture. Reutilization was achievable for more reaction cycles. The immobilization efficiency was
96.03 % and reusable activity of immobilized cellulase was 51% after 10 reaction cycles. It is concluded that the immobilized cellulase can be applied for multiple reuse of biotransformation reaction cycle.

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