Immobilization of diastase on PVA- CoFe$_2$O$_4$ nanocomposite film for improving stability and recycling

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Abstract. Enzyme immobilization on a suitable support is one of the strategies used to improve enzyme stability and recovery. In present communication, PVA-CoFe$_2$O$_4$ nanocomposite film has been used as a support to develop highly active, stable and magnetically separable immobilized diastase. For preparing magnetically separable support, first Cobalt ferrite nanoparticles have been prepared by using co-precipitation method. Prepared nanoparticles have been dispersed in polyvinyl alcohol (PVA) solution to get a PVA-CoFe$_2$O$_4$ film. The detail characterization of cobalt ferrite nanoparticles and PVA-CoFe$_2$O$_4$ film has been carried out. Enzyme diastase has been immobilized on the PVA-CoFe$_2$O$_4$ film and the catalyst activity of immobilized enzyme has been tested in hydrolysis of starch to maltose. The stability of immobilized diastase has found to be better than free enzyme. Reusability study of the immobilized diastase has been carried out. Improved stability and reusability of immobilised diastase offer the promising applicability as magnetically separable biocatalyst for industry.

Keywords: PVA - CoFe$_2$O$_4$ Nano composite, Immobilized enzyme, Magnetically separable biocatalyst

1. Introduction
Designing catalysts that will meet the demands of green and sustainable chemistry is a key task for development of greener and environmentally sound synthetic protocols [1]. Biocatalysts particularly enzymes satisfy most of the requirements of the sustainable and green catalyst and therefore are expedient substitutes for traditional catalysts. These biocatalysts offer significant advantages like high specificity [2], mild reaction conditions [3] and environmental friendliness [4]. Though enzymes have ability to perform most complex chemical processes under environmentally benign conditions, their use in chemical industries is still limited. The problems associated with use of enzyme as industrial biocatalysts are enzyme instability at high temperature and in toxic solvents, difficulties with recovery from reaction mixture and high cost of isolation [5]. The enzyme recovery and reuse are an important aspect for economically feasible processes that may compensate the high cost of enzyme. Enzyme immobilization is one of the strategies used to improve enzyme stability and recovery. Enzyme immobilization is a technique in which catalysts are attached to a solid support that is insoluble in the reaction mixture [6]. Binding of enzymes to solid supports not only improves the stability but also convert catalytic systems into heterogeneous form which makes easy
separation of the biocatalytic system from the reaction mixture. Thus, main goals of immobilization of enzymes are improved enzyme stability, easy recovery and reuse. In this context, development of suitable immobilization technique for a particular enzyme is an interesting area of research. Designing a suitable immobilization technique is a key task in development of industrial biocatalysts which requires multidisciplinary approach. Various immobilization techniques viz. adsorption, covalent binding, encapsulation, cross-linking and entrapment have been developed [7]. Range of materials like inorganic, organic, hybrid and composite materials have been used as stable and efficient supports for biocatalysts [8]. Recently, use of magnetically separable nanoparticle (MNP) as a support for enzyme immobilization has been studied extensively in view of an easy recovery of immobilized enzyme and possible reuse. MNPs provide large surface area and hydroxyl group on their surface allows easy modification of the surface and possibility of strong binding with the enzyme. MNPs are advantageous as the biocatalytic system immobilized on magnetic support can be easily separated using an external magnet [9]. Functionalized magnetic nanoparticles including graphene-Fe₃O₄[10], silica- Fe₃O₄[11], and polymer grafted- Fe₃O₄[12, 13] have been used as support. Variety of enzymes viz. Lipase [14], glucose oxidase [15], trypsin [16], Amylase [17] etc. have been immobilized on functionalized magnetic nanoparticles.

In recent years there has been growing interest in the use of commercially available polymeric films due to their easily tunable properties, good porosity and a large surface area for attachment of variety of enzymes. The well-defined pore sizes and structure of polymeric films facilitate the immobilization of biomolecules not only on the surface of the support but also in its pores [18]. Poly (vinyl alcohol), poly(ethylene glycol), polyurethane and poly (vinylidene fluoride) has been used to prepare polymeric membrane for enzyme support [19,20,21].

In present communication we have developed a Poly vinyl alcohol (PVA) and CoFe₂O₄(MNP) nanocomposite film to combine the advantages of both the types of supports. The PVA-MNP film has been used to immobilize diastase. The resulting biocatalytic system has been tested in hydrolysis of starch to glucose and the stability and activity of immobilized enzyme has been compared with free enzyme.

2 Material and Methods
2.1 General Remarks
Fourier transform-infrared spectra of the catalysts reported in this study were recorded on Shimadzu 8300 FTIR at ambient conditions. The spectra were recorded between 400 and 4000 cm⁻¹. The XRD patterns of the catalytic samples were taken using PHILIPS (PW3710) X-ray diffractometer with Cu Kα radiation (λ = 1.5424 Å). SEM micrographs of samples were taken on JEOL- JEM - 6360 microscope. The room temperature (300 K) magnetic properties of the prepared CoFe₂O₄ nanoparticles were investigated by the VSM technique in the range of approximately –15 to +15 kOe.

2.2 Reagent and materials
Ferric chloride anhydrous LR (FeCl₃) and Polyvinyl alcohol LR (PVA) were supplied by THOMAS BAKER (Mumbai, India). Cobalt (II) chloride extra pure (Hexahydrate) (CoCl₂·6H₂O), Sodium hydroxide (NaOH), Starch soluble (Ex potato) (C₆H₁₀O₅)n were supplied by MOLYCHEM (Mumbai India).

2.3 Preparation of cobalt ferrite nanocatalyst
CoFe₂O₄ was synthesized by the reported method [22]. In a typical procedure, to a well stirred solution of stoichiometric amount of FeCl₃·7H₂O and CoCl₂·6H₂O in water (in 2:1 molar proportion) was added drop wise aqueous 4M solution of sodium hydroxide. The resultant precipitate was filtered and washed several times with de-ionized water to remove chloride ions. It was dried under vacuum at 60°C for six hours. The resultant cobalt ferrite particles were sintered between 800- 1000°C for 2 hours.
2.4 Preparation of PVA–Cobalt ferrite film

CoFe$_2$O$_4$ nanoparticles prepared using co-precipitation method were dispersed into 20 ml of PVA (50 mg/ml) solution in distilled water. The hot solution was poured on petri plate to get uniform film and allowed it to dry for 2 days.

2.5 Immobilization of Diastase on PVA–Cobalt ferrite film

30 mg of diastase (Himedia) enzyme diluted in 3 ml saline was poured uniformly over PVA–Cobalt ferrite film. The film was allowed to stand for 24 hours at temperature 4 °C. After 24 hours, the film was washed with saline. Protein content in washing was checked by Lowery method [23]. Similar procedure had been used for 20 mg, 40 mg, 80 mg and 120 mg enzyme concentration.

2.6 Testing of Enzyme Activity

The resulting biocatalytic system was tested in hydrolysis of starch to glucose. After washing, 1 ml of 1% starch and acetate buffer (pH 4.6, 0.2 M) were poured on film and solution was incubated at 37°C for exactly 15 min. The solution was collected and digested reducing sugars were tested with DNSA method. Enzyme activity on same film was rechecked after 10 days.

2.7 Comparison with free enzyme

To compare the activity of immobilized enzyme, 30 mg free enzyme and 30 mg enzyme immobilized on PVA–Cobalt ferrite film were tested in hydrolysis of starch to maltose.

3. Result and discussion

The Fourier Transform Infrared Spectroscopy was performed to confirm the formation of spinel ferrite (Figure 1). The FTIR spectrograph of cobalt ferrite sample [Fig. 1] shows two characteristic absorptions at 583 cm$^{-1}$ and 464 cm$^{-1}$ for octahedral and tetrahedral metal–oxygen stretching in spinel ferrites.

![Figure 1. IR spectrograph of CoFe$_2$O$_4$](image)

The phase purity of the prepared catalyst was analysed by studying powder X-ray diffraction pattern. The powder XRD patterns for Cobalt ferrite NPs and PVA-Cobalt ferrite film are displayed in Figure 2. In case of CoFe$_2$O$_4$, Fig. 2(a) all the detectable peaks are indexed as CoFe$_2$O$_4$ with an inverse spinel structure and are in good agreement with the standard data (JCPDS NO. 22-1086). The crystallite size of the sample was found to be 40 nm.
Figure 2. (a) XRD pattern of CoFe$_2$O$_4$–MNPs

Figure 2. (b) XRD pattern of PVA - CoFe$_2$O$_4$ film

Figure 3 depicts the microstructure of the cobalt ferrite and PVA - CoFe$_2$O$_4$ film sample. The SEM topographs of CoFe$_2$O$_4$ catalyst shows that all the grains are in uniform size and distributed uniformly over the surface. The SEM image of PVA - CoFe$_2$O$_4$ film depicts rough and granular surface. From SEM images it can be concluded that PVA - CoFe$_2$O$_4$ film provides large surface area for binding of enzymes.

Figure 3. (a) SEM image of CoFe$_2$O$_4$ – MNPs

Fig.3 (b) SEM image of CoFe$_2$O$_4$ – MNPs film

Figure 4. Hysteresis loop for CoFe$_2$O$_4$
Figure 4 shows hysteresis loops of the CoFe$_2$O$_4$– MNPs and confirms the ferrimagnetic behaviour of the particles with saturation magnetization value ($M_s$) of 70.10 emu / gm which is an important property for easy separation of the catalyst using an external magnet for its reuse.

To check the maximum retention capacity of PVA – Cobalt ferrite film, increasing amount of enzyme viz. 20 mg, 30 mg, 40 mg, 80 mg, and 120 mg were immobilized on PVA – Cobalt ferrite film. Each film was tested for enzyme activity and washings were tested to check protein loss after immobilization. Fig. 5 shows enzyme activities of different concentration of diastase immobilized on PVA-Cobalt ferrite film and enzyme activity of each film after 10 days.

![Graph showing enzyme activities of different concentration of diastase immobilized on PVA-Cobalt ferrite film and enzyme activity of each film after 10 days.]

Increasing enzyme concentration showed gradual increase in enzyme activity. At 80 mg enzyme concentration, almost 5fold increase in enzyme activity was observed as compared to 20 mg enzyme concentration. Though enzyme activity increased even after 80 mg to 120 mg, the washings of films showed presence of protein. At 120 mg 0.6818±0.07 mg protein loss was observed. It was concluded that up to 80 mg the enzyme gets trapped into pores and get retained on the support. However beyond 80 mgs the enzyme remains on the surface and hence resulted in protein losses. Up to 80 mg 100% immobilization was observed (protein content was nil) due to entrapment but above 80 mg protein losses was observed. Thus, maximum rate of adsorption 1.1658 mg/cm$^2$ was observed for diastase.

To compare activity of immobilized enzyme with free enzyme, 30 mg free enzyme and 30 mg enzyme immobilized on PVA – Cobalt ferrite film were tested in hydrolysis of starch to maltose. It was found that 30 mg free enzyme showed 150.11±12.3 units per mg activity while the immobilized enzyme showed 154.66±11.2 units per mg activity. Thus, activity of immobilized enzyme is comparable with free enzyme.
Reusability Study of immobilized enzyme: Main aim behind the physical immobilization of enzyme is reusability. In this case plates were stored at 4°C and reused after 10 days. It was found that at lower concentrations almost 80 to 90% activity of enzyme has been retained while at higher concentration i.e. 80mg and 120 mg almost 40% loss of enzyme activity has been observed. Porosity of film might be protecting the enzyme from washing loss at lower concentrations. Reusability study suggested that up to 40 mg immobilized enzyme has appropriate activity, stability and reusability up to 5 runs (Fig.6).

4. Conclusion

Highly active, stable and magnetically separable immobilized diastase has been developed using PVA-Nano CoF2O4 film as support. The method developed for immobilization of enzyme is effective and the stability of immobilized diastase has found to be higher than free enzyme. The activity of diastase immobilized on PVA – nano Cobalt ferrite film has found to be comparable with free enzyme. The immobilized diastase can be reused effectively over period of 10 days.

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