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Immobilization of α-amylase onto cellulose-coated magnetite (CCM) nanoparticles and preliminary starch degradation study

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ABSTRACT

The present work is focused on the preparation of cellulose-coated magnetite (CCM) nanoparticles obtained by coagulation of aqueous solution of cellulose containing magnetite nanoparticles. The size of the CCM nanoparticles, as obtained by TEM analysis, was found to be in the range of 2.5–22.5 nm. The overall thermal degradation of plain cellulose and CCM particles, when studied in the temperature range of 30–600 °C, was nearly 48 and 38% respectively. The XRD analysis also confirmed the formation of CCM nanoparticles. The molecules of enzyme amylase were attached to the cellulosic surface by a chemical route involving periodate induced oxidation of cellulose to dialdehyde, followed by covalent attachment with enzyme molecules. The CCM nanoparticles, containing 16 and 28 wt% of cellulose, demonstrated maximum attachment of 9.1 and 16.2 mg of amylase/g of CCM particles for 0.05 M concentration of periodic acid. The uptake of amylase by CCM (28) particles followed Langmuir isotherm model with maximum uptake (q_{max}) of 18.2 mg/g CCM (28) particles. Finally, the degradation of starch by amylase-immobilized CCM (28) nanoparticles was also investigated. The values of Michaelis constants K_M and maximum degradation rate r_{max} were found to be 7.5×10^{-7} μmol ml^{-1} and 0.04×10^{-6} μmol^{-1} g^{-1} min^{-1} respectively. The enzyme-immobilized nanoparticles were investigated for their starch degrading capacity under optimal conditions.

1. Introduction

Polymer-coated magnetic nanoparticles possess both, potent magnetic susceptibility and a reactive polymer surface with large surface area, on which functional biomolecules, such as enzyme [1], plasmid DNA [2], bioactive molecules or drugs [3], human cells [4], oligonucleotides [5], etc., can be immobilized. The magnetic polymer nanoparticles are usually composed of a magnetic core surrounded by a polymer shell. The magnetic part is often magnetite nanoparticles while polymer shell, composed of polymers such as silica [6], chitosan [7], hyaluronic acid [8], polyethylene glycol [9], etc., provides favourable functionality to magnetic particle. The basic requirement for using magnetic polymer nanoparticles in biomedical and therapeutic applications is that the polymer coating must be biocompatible, and non-toxic. On the other hand, for industrial applications the polymer must not only be eco-friendly but it should also be cheaper and easily available and should have low processing cost.

Enzyme, immobilized on a polymer surface, has many advantages over free enzyme including repeated or continuous use, easy separation of the product from reaction system, easy recovery of enzyme, and enhancement in enzymatic stability. However, using the enzyme-immobilized magnetic particles makes the recovery of enzyme more effective and easy as there is no need to apply high speed centrifugation [10], but a moderate magnetic filed causes almost complete and easy removal of enzyme from the reaction system. Therefore, enzyme immobilized onto surface of magnetic nanoparticles is preferred to native enzyme for the purpose of using in industrial applications now-a days. Recently, Wang et al. [11] have reported immobilization of Pycnoporus sanguineuslaccase by magnetic chelator particles via metal affinity adsorption. The immobilized laccase could be easily operated magnetically and it exhibited remarkably improved catalytic activity and stability. Similarly, mimetic enzyme, hemin has been successfully immobilized on silica-coated magnetic nanoparticles and investigated for its peroxidase activity [12]. The magnetic silica nanoparticles supported hemin showed long term stability toward temperature and good reusability. Similarly, Schultz et al. [13] have studied activity of lipase, immobilized on micro-magnetic particles, using spectrophotometry.

Cellulose is one of the most abundant renewable resources and is biodegradable, eco-friendly and non-toxic polymer [14]. However, due to highly crystalline structure and presence of strong inter and intramolecular hydrogen bonding interactions among hydroxyl groups, it is practically insoluble in common solvents like

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water [15]. Therefore, it is not possible to prepare cellulose–metal nanoparticles composite materials. A thorough survey of the literature available also reveals that cellulose coated magnetite nanoparticles have not been fabricated, probably due to insolubility of cellulose. Recently, Ruan et al. [16] reported that cellulose could easily be dissolved in NaOH/thiourea system. Making use of their findings, we have developed a unique approach to prepare cellulose-coated magnetite (CCM) nanoparticle. The surface of the CCM nanoparticles, produced has been modified via periodic acid induced oxidation to attach enzyme molecules covalently. The enzyme immobilized CCM particles have been investigated to carry out degradation of starch in aqueous medium. To the best our knowledge, immobilization of α-amylase onto the surface of magnetite-coated cellulose is being reported for the first time.

2. Experimental

2.1. Materials

Cellulose powder, starch, ferrous chloride, ferric chloride, sodium hydroxide were purchased from Hi-media Laboratories, Mumbai, India. Enzyme α-amylase was received from S.D. Fine chemical, Mumbai, India and used as received. The double distilled water was used throughout the investigations.

2.2. Method

2.2.1. Synthesis of magnetite nanoparticles

Magnetite nanoparticles (Fe_3O_4) were prepared by chemical co-precipitation of Fe^{2+} and Fe^{3+} ions in aqueous solution of sodium hydroxide, followed by treatment under hydrothermal conditions [17]. In brief, Fe(II) and Fe(III) were dissolved in acidified water in 1:2 molar ratio and to this, aqueous solution of NaOH (30%, w/v) was added drop-wise under constant stirring at 40 °C at a controlled pH 10–10.4. The suspension was heated at 90 °C for one hour under continuous stirring and magnetite nanoparticles so obtained were separated by centrifuging several times in water and then in ethanol at 200 rpm. The purification step was used to remove impurities from Fe_3O_4 nanoparticles. The particles were finally dried in vacuum at 70 °C.

2.2.2. Preparation of cellulose-coated magnetite nanoparticles

The CCM nanoparticles were obtained by coagulation of magnetite containing cellulose solution in ammonium sulfate. In brief, 4 g of cellulose was added to distilled water, followed by dissolution of 6 g of NaOH and 4 g of urea. The above suspension was put at −5 °C for 12 h, and then stirred vigorously at 10 °C to obtain almost transparent solution. To this aqueous solution of cellulose, pre-calculated quantity of magnetite nanoparticles was added under constant stirring to ensure homogeneous mixing. The suspension was allowed to be dropped into 8% (w/v) ammonium sulfate solution under vigorous stirring. The resulting CCM nanoparticles were filtered, washed several times with distilled water and then dried at 60 °C in oven (Tempstar, India). In all, we prepared two CCM nanoparticles samples with cellulose content of 16 and 28% (w/v) respectively. We shall designate them as CCM (16) and CCM (28) respectively.

2.2.3. Oxidation of cellulose coating of CCM nanoparticles [18]

A 5 g quantity of cellulose-coated magnetite nanoparticles was put in 250 ml of 0.03 M periodic acid. The pH of the solution was adjusted to 4.0, followed by heating in water bath at 80 °C under constant stirring for 15 h. The CCM nanocomposite particles were then filtered, washed with distilled water and then dried in electric oven (Tempstar, India) at 40 °C.

2.2.4. Immobilization of α-amylase to oxidized CCM nanoparticles

A 1.0 g quantity of dry oxidized CCM nanoparticles was immersed in 30 ml aqueous solution of α-amylase of known concentration and incubated in water bath at 50 °C under constant stirring for 30 min. The samples were taken out and washed with distilled water to remove unreacted enzyme retained on the cellulose coating, and then dried in a dust-free chamber at 35 °C.

2.2.5. Quantitative determination of enzyme

Starch solutions of definite concentration were allowed to be degraded by enzyme solutions of varying concentrations and the sugar released was estimated spectrophotomertically using DNS method [19]. Now a calibration curve was plotted between absorbance and enzyme content, which yielded a fair linear plot. Using this standard curve, concentrations of enzyme immobilized onto the cellulose surface were obtained by degrading starch and measuring the absorbance of the solutions. The amount of enzyme bound covalently to the oxidized surface of cellulose coating of CCM nanoparticles was determined in mg g^{-1} using following expression:

$$\frac{x}{m}\,(\text{mg g}^{-1}\text{CCM particles}) = \frac{(C_0 - C_e) \times v}{d \times 100}$$

where C_0 and C_e are the initial and the equilibrium concentrations of enzyme solutions (in mg/100 ml), v is volume of reaction medium (in ml) and d is dry weight of CCM particles taken in the reaction system for enzyme immobilization.

2.3. FTIR spectroscopy analysis

The FTIR spectra of cellulose, cellulose-coated magnetite and amylase bound CCM nanoparticles was recorded by FTIR spectrophotometer (Shimadzu, Model no. 8400S PC spectrophotometer) using KBr.

2.4. Transmission electron microscopy (TEM) analysis

TEM image was obtained by employing JEM-2010 microscope under 200 kV. The sample for observation of TEM was prepared by placing three drops of suspension, prepared in acetone, onto a 3 mm copper grid.

2.5. XRD analysis

XRD analysis was performed with a Rikagu diffractometer (Cu radiation, λ = 0.1546 nm) running at 40 kV and 40 mA.

2.6. Thermogravimetric analysis (TGA)

TGA was performed using a thermogravimetric analyzer (Mettler Toledo TGA/SDTA 851®) controlled by STAR® software (Mettler Toledo GmbH, Switzerland). About 10 mg of powder sample was placed in ceramic crucible and analyzed over the temperature range of 30–600 °C at the heating rate of 10 °C min^{-1} under dry flow of N_2 at the rate of 30 ml/min.

3. Results and discussion

3.1. Formation of cellulose-coated magnetite nanoparticles

The overall preparation of CCM nanoparticles consists of following three steps: (1) dissolution of cellulose in NaOH/urea system, (2) uniform mixing of magnetite into cellulose solution and finally, (3) coagulation of above suspension in ammonium sulfate to yield CCM nanoparticles. These steps may be explained as follows:

(1) When cellulose is dissolved in NaOH/urea solvent system, the intermolecular hydrogen bondings between O'_3 and H–O_6, and O'_5 and H–O_6 are broken. In addition, intramolecular hydrogen bonding between O'_6 and HO_2 are also destroyed. As a result, free CH_2OH groups undergo conformational transformation from trans-gauche (t_g) to gauche-trans (g_t) thus resulting in formation of new intramolecular $O_6H\cdots O'_2$ and intermolecular $O_6\cdots OH$ bondings along the cellulosic chains [20]. This ultimately causes in dissolution of cellulose.
(2) When magnetite nanoparticles are mixed into aqueous solution of cellulose, a uniform suspension is obtained. The binding of Fe(II) part of magnetite with electron rich O atoms of cellulose macromolecular chains contributes to uniform mixing.
(3) When the uniform suspension is allowed to be dropped into coagulating bath, magnetite nanoparticles with cellulose coatings are obtained.

3.2. Covalent attachment of enzyme on the CCM's surface

When cellulose-coated magnetite nanoparticles are treated with periodic acid, cellulose is oxidized to dialdehyde. On further treatment of dialdehyde with α-amylase, the enzyme is covalently immobilized on the cellulose surface via coupling reaction. The whole reaction is well shown in Fig. 1.

3.3. Characterization of CCM nanoparticles

3.3.1. FTIR spectral analysis

The FTIR spectrum of cellulose, CCM and enzyme amylase binded CCM particles as shown in Fig. 2. In Fig. 2(A) presence of a strong broad band in the range of 3200–3400 cm^{-1} corresponds to stretching vibration of N–H and OH groups where as peaks obtained at 2524 and 2893 cm^{-1} refers to the C–H stretching vibrations. The band in the region of 1758 cm^{-1} refers to C=O stretching whereas the peak at 1442 cm^{-1} refers to C–O stretching of plain cellulose. Same peaks are also observed in the spectrum of CCM nanoparticles (Fig. 2(B)) along with an additional peak at 509 cm^{-1} which corresponds to Fe–O binding, Fig. 2(C) reveals additional peak at 3834 cm^{-1} due to $-NH_2$ stretching, thus confirming the presence of amylase.

3.3.2. XRD analysis of CCM nanoparticles

The XRD pattern of cellulose-coated magnetite nanoparticles has been well depicted in Fig. 3. It is clear that XRD pattern consists of characteristic peaks of both the magnetite and the cellulose. The peaks appearing at $d = 3.07$, 2.78, 2.53, 2.33 and 2.03 resemble very closely with the theoretical value of 2.97, 2.78, 2.64, 2.51, 2.53 and 2.10 respectively [21]. This confirms the presence of magnetite within the CCM nanoparticles. A close look at XRD pattern also reveals that the peak, corresponding to $2\theta = 11.8^\circ$ is not so sharp thus suggesting that regeneration of cellulose (i.e. dissolution in NaOH/urea solvent system followed by coagulation in the ammonium persulfate solution) results in weaker H-bonding interactions between $(1\,\bar{1}\,0)$ planes in CCM nanoparticles [22]. On the other hand, peak corresponding to $2\theta = 19.1^\circ$ is comparatively sharp, thus suggesting that cellulose molecules, after regeneration, are re-arranged more easily on (1 1 0) plane [23]. In fact, regenerated cellulose has been observed to possess less-crystalline structure as also pointed out by other workers [24].

3.3.3. TEM analysis

Fig. 4 shows the TEM image of the cellulose-coated magnetite nanoparticles. It is clear that most of the particles are lying in the narrow range of particle size and have almost spherical shape. We also carried out particle size analysis by selecting a number of particles from arbitrarily chosen areas in the TEM image. It is clear that

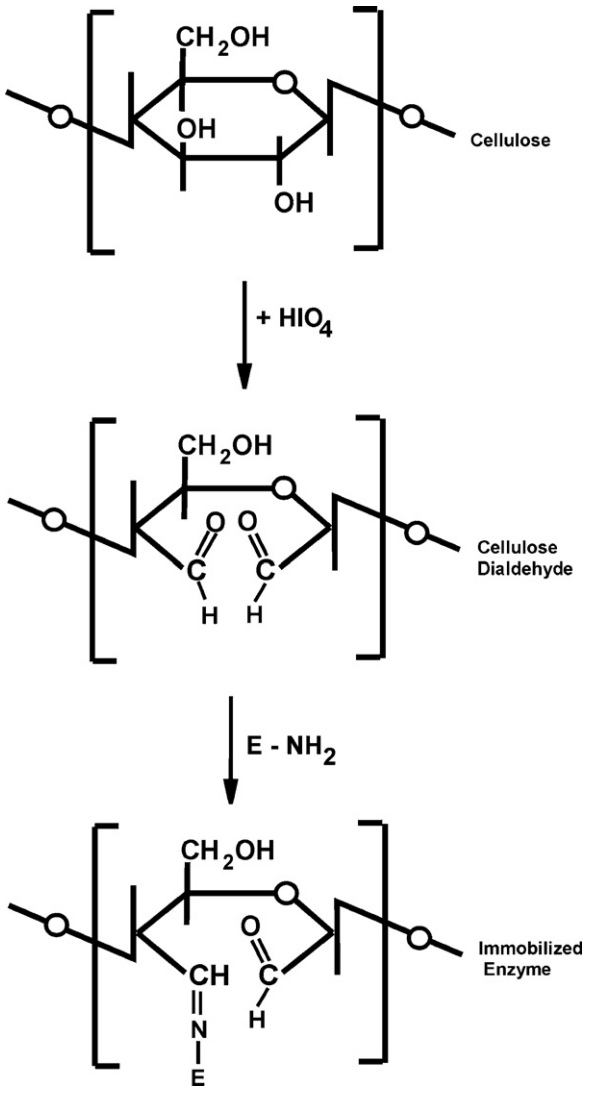

Fig. 1. Scheme of formation of cellulose coated magnetite nanoparticles.

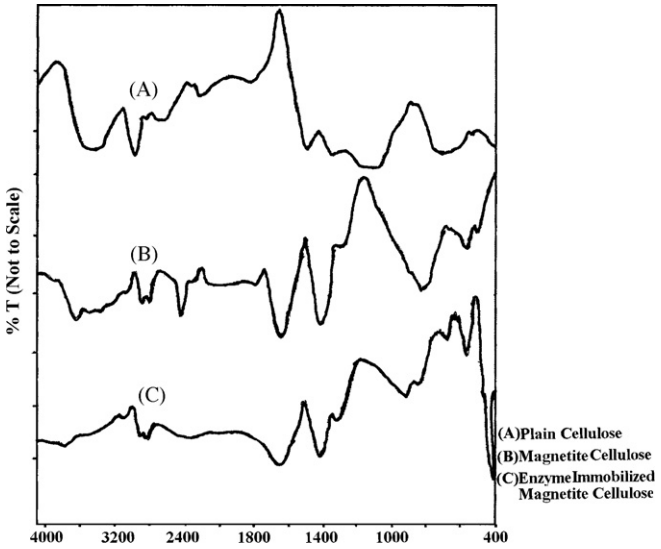

Fig. 2. FTIR spectra of (A) plain cellulose,(B) CCM particles and enzyme binded CCM particles.

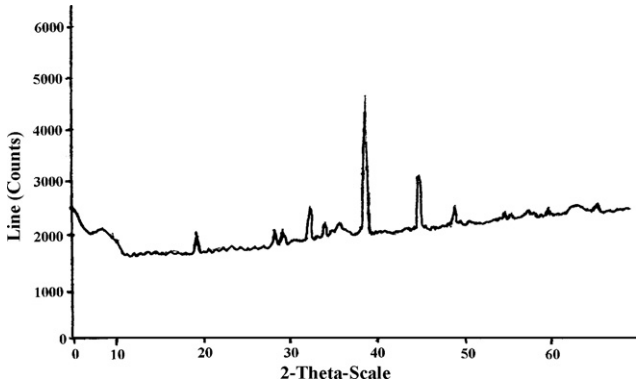

Fig. 3. XRD diffraction pattern of (A) plain hydrogel and (B) magnetite-loaded hydrogel.

the average range of their diameter lies between 2.5 and 22.5 nm and nearly 50% of particles fall with their average diameter 7.5 nm. Such small sized CCM nanoparticles are desirable as they provide in large surface area available for immobilization of enzyme.

3.3.4. TGA analysis

Since magnetite nanoparticles have fair thermal stability, the cellulose-coated magnetite nanoparticles are expected to be more stable as compared to cellulose. In order to investigate this, we carried out thermogravimetric analysis of the plain cellulose and the cellulose-coated magnetite nanoparticles. A comparative depiction of thermograms has been given in Fig. 5. It is clear that in the temperature range of 30–130 °C, the initial weight losses for plain cellulose (PC) and CCM are nearly 9.0 and 15.0% respectively. This may simply be due to the release of moisture from the samples. The greater weight loss for CCM nanoparticles is due to the presence of the hydrophilic magnetite nanoparticles which contributes to a great moisture loss. However, in the second phase, PC and CCM suffer weight losses of nearly 21 and 8% respectively in the temperature range of 130–240 °C. This indicates greater thermal stability of later due to presence of magnetite nanoparticles. Moreover, in the final phase, the PC suffers percent weight loss of nearly 14 in the temperature range of 240–400 °C whereas the CCM exhibit nearly 6% weight loss in the same temperature range. Finally, the total weight loss for PC and CCM up to 600 °C is found to be nearly 48 and 37% respectively, thus confirming that cellulose-coated magnetite nanoparticles posses greater thermal stability as compared to plain cellulose. This may also be taken as evidence for presence of magnetite within cellulose coated magnetite nanoparticles. Similar three phase thermal analysis has also been reported elsewhere [25].

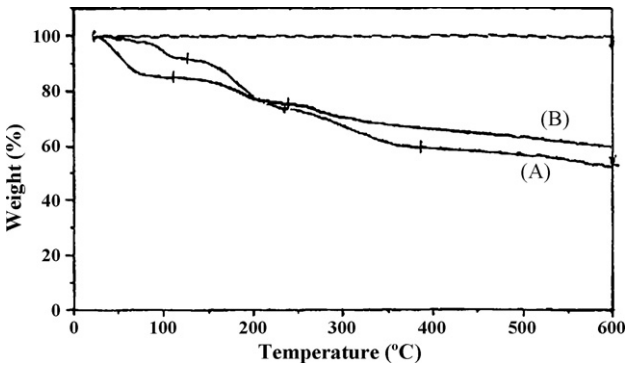

Fig. 5. TGA of (A) plain cellulose and (B) CCM particles.

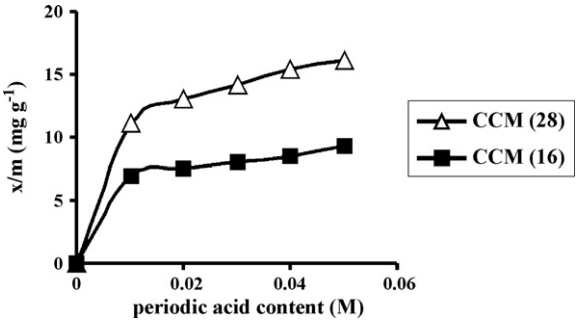

Fig. 6. Effect of periodic acid content on enzyme immobilization.

3.4. Effect of periodic acid content on enzyme immobilization

The covalent attachment of α-amylase, as discussed previously, is mainly due to coupling reaction between amine groups of enzyme and aldehyde groups of cellulose dialdehyde present as coating material on magnetite nanoparticles. We prepared two CCM samples having different cellulose content, namely CCM (16) and CCM (28) (see Section 2) and put them in different solutions of periodic acid, in the range 0.01–0.05 M for transformation into cellulose dialdehyde, and then treated with enzyme solutions of same concentration for covalent attachment. The results, as depicted in Fig. 6 clearly indicate that amount of enzyme immobilized per g of CCM nanoparticles (i.e., x/m) increases with the concentration of periodic acid. This may simply be attributed to the fact that with the increase in periodic acid content, number of dialdehyde groups on cellulose molecules increase. In other words concentration of cellulose–dialdehyde in cellulose coating increases, thus resulting in attachment of more enzyme molecules.

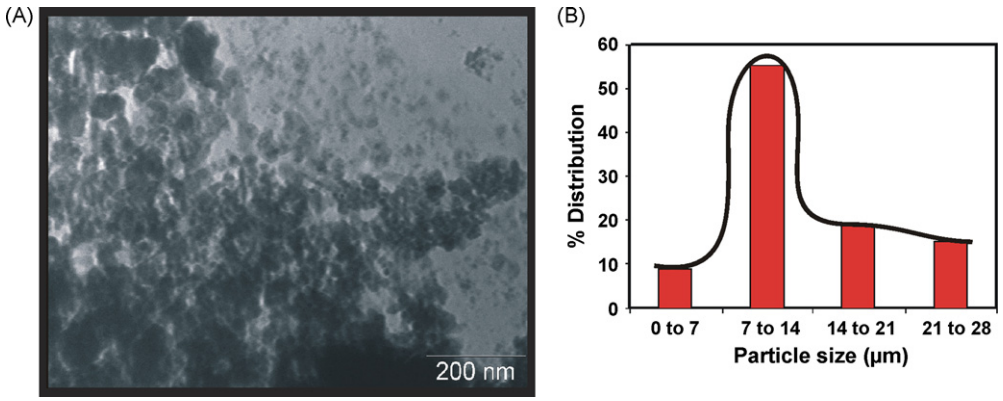

Fig. 4. (A) TEM image of CCM nanoparticles with SAED pattern in inset and (B) particle distribution graph.

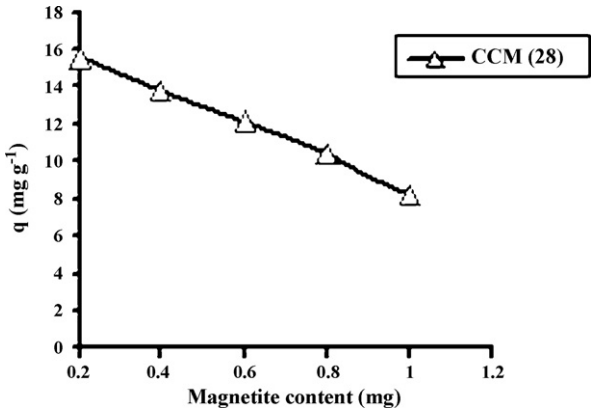

Fig. 7. Effect of magnetite content in CCM nanoparticles on enzyme immobilization.

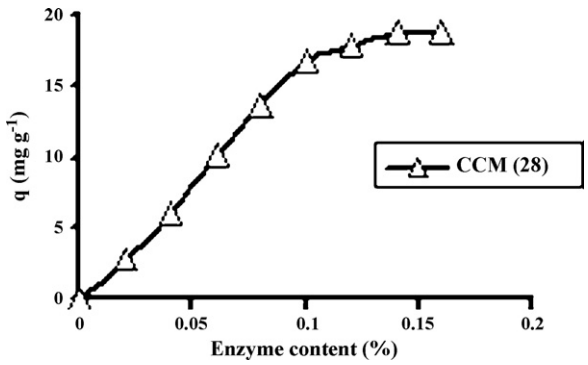

Fig. 8. Effect of enzyme concentration on immobilization.

However, the amount of enzyme attached attains almost saturation value when the periodic acid content exceeds beyond 0.05 M. It is also clear from Fig. 6 that for a given concentration of periodic acid, more enzyme is attached on the surface of the sample CCM (28) as compared to the sample CMC (16), which is simply due to the fact that sample CCM (28) contains more cellulose and hence produces greater number of dialdehyde groups responsible for enzyme attachment. For the optimum concentration of 0.05 M of periodic acid the amount of amylase attached per gram of CCM (16) and CCM (28) nanoparticles was 9.1 and 16.2 mg respectively.

3.5. Effect of magnetite content in CCM on enzyme immobilization

We prepared a number of samples by mixing different amounts of magnetite in cellulose solutions of same concentration, followed by their coagulation in ammonium sulfate solution. These samples were allowed to react with periodic acid solution of same concentration, followed by immobilization of enzyme. Fig. 7 shows the effect of variation in amount of magnetite present in cellulose dope on concentration of amylase immobilized. It is clear that as the amount of magnetite (expressed as percent of magnetite mixed into cellulose solution) increases in the CCM samples, the amount of enzyme immobilized decreases. This is simply due to of the fact that with the increases in content of magnetite, the relative concentrations of cellulose in the dope decreases. Therefore, when these solutions are dropped into coagulation bath, the content of cellulose available in surface coating in resulting CCM nanoparticles decreases. This ultimately results into production of lesser number of dialdehyde groups on periodate-induced oxidation and hence causing in covalent attachment of fewer enzyme molecules on CCM nanoparticles. Therefore, it may be concluded that if the magnetite content in the dope (i.e. cellulose solution) increases, the amounts of enzyme immobilized decreases.

3.6. Effect of enzyme concentration on immobilization

Fig. 8 shows the effect of enzyme concentration in the solution on its immobilization onto the surface of CCM (28) nanoparticles. It is quite clear that with the increase in concentration of enzyme solutions, more and more enzyme molecules get attached via covalent linkage onto the oxidized surface of the CCM (28) nanoparticles. However, when the enzyme concentration exceeds 0.15% (w/v), the amount of enzyme immobilized per gram of CCM (28) nanoparticles attains saturation value, i.e. 18.2 mg amylase/g CCM (28) nanoparticles. The observed finding may simply be explained by the fact that in the solution with higher enzyme concentrations, the chances of their attachment with dialdehyde groups increases, thus resulting in enhancement in enzyme immobilization. However, when the amount of enzyme in the solution exceeds 0.16% (w/v) the reaction sites in cellulose coatings get fully occupied by the enzyme molecules thus showing maximum enzyme uptake. Finally, the equilibrium uptake data was applied on the Langmuir sorption isotherm model [25].

$$q = \frac{q_{\max} \cdot bC_e}{1 + bC_e}$$

where q is amount of sorbate adsorbed per unit mass of sorbent, $q_{\max}$ is the maximum metal uptake per unit mass of sorbent (i.e. saturation), C_e is the equilibrium concentration of sorbate in the solution and b is Langmuir constant related to energy of sorption which reflects the affinity between sorbent and the sorbate. The results, as shown in Fig. 9 clearly shows L_2-type nature of the sorption curve, [27] thus indicating strong tendency of enzyme molecules to bind with the sorbent particles (i.e., CCM [28] nanoparticles with dialdehyde cellulose as coating material).

3.7. Enzymatic degradation of starch by CCM nanoparticles

Covalent binding is very effective in retaining the enzyme and can achieve high activity after immobilization, if amino acid residues that are covalently bound with the support materials are not involved in the active site or the substrate binding site. However, there are reports about the decrease in the activity due to immobilization [28]. The drop in activity can be attributed to steric hindrance in the immediate vicinity of the enzyme molecules. The hindrances are probably caused by the shielding effect of the substrate and by the excessive packing of enzyme. For example, after the covalent attachment of amylase on the glass beads, the immobilized enzyme retained 72% of specific activity. In the present study, since the substrates are nanometer sized cellulose-coated mag-

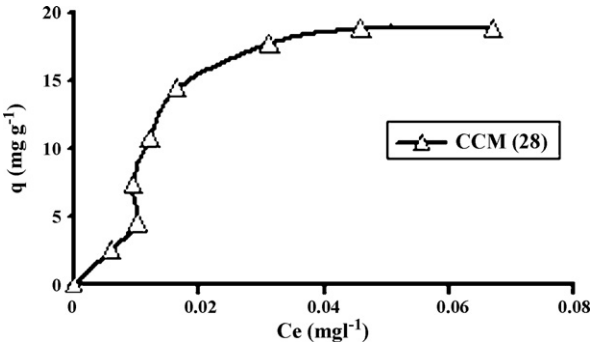

Fig. 9. Langmuir L_2 nature of the sorption curve.

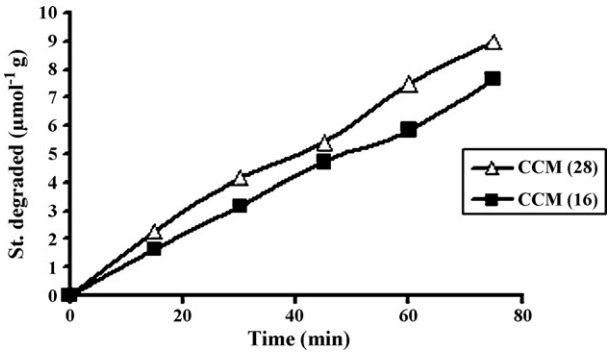

Fig. 10. Enzymatic degradation of starch (St.) by CCM nanoparticles.

netite nanoparticles, the chances of hindrance by substrate are rare. However, this requires a detailed investigation. Fig. 10 shows the kinetics of degradation of starch by equal quantities of CCM (28) and CCM (16) nanoparticles (i.e. 1.5 g for each sample) at 37 °C. The results clearly indicate that amount of starch degraded at different time intervals is more for sample CCM (28) as compared to the sample CCM (16). The reason is that sample CCM (28) contains more enzyme immobilized on the surface due to the relatively higher cellulose content as already described previously. The initial starch degradation rates, when calculated from the initial linear portion of the plots, were found to be 0.128 and 0.105 μg min^{-1} g^{-1} CCM particles respectively. It is also here noteworthy that it required nearly 110 min for obtaining complete degradation of starch. After the degradation was over, the CCM nanoparticles were completely recovered by just introducing a bar magnet in the degradation medium. In this way, enzyme-loaded CCM nanoparticles can be removed easily from the degradation system at any moment when required. This establishes the superiority of the present enzyme-immobilized nanoparticles over the convectional non-magnetic enzyme-immobilized polymeric substrate which need high speed centrifugation followed by filtration for removing enzyme from the degradation system [26].

3.8. Kinetics of enzymatic degradation

The kinetics of starch degradation by α-amylase immobilized CCM nanoparticles was studied by taking starch solutions of varying concentrations. The starch solutions, in the range of 0.16–0.8% (w/v), were degraded by same amounts of enzyme-attached CCM nanoparticles in the buffer medium of pH 7.4 at 37 °C. The initial slopes of amount of starch degraded versus time profiles were used calculate initial starch degradation rates. Finally the reciprocals of starch concentration were plotted against reciprocals of initial degradation rates (see Fig. 11) following one of the known

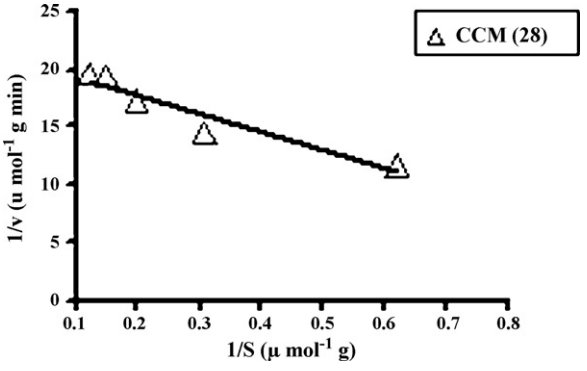

Fig. 11. Evaluate the values of Michaelis constant K_M and the maximum rates r_{max}.

presentations of MM kinetics [29]

$$\frac{1}{r_1} = \left[\left(\frac{1}{r_{max}}\right) + \left(\frac{K_M}{r_{max[S]}}\right)\right]$$

where r_1 denotes the degradation rate, r_{max} is the maximum rate of degradation, and [S] represents the substrate concentration. Using the slopes and intercept of the linear plot obtained, Michaelis constant K_M and the maximum degradation rate r_{max} were evaluated and were found to be 7.5×10^{-7} μ mol ml^{-1} and 0.04×10^{-6} μ mol^{-1} g^{-1} min^{-1} respectively. The relatively smaller value of K_M is indicative of the higher substrate–enzyme affinity [19].

4. Conclusions

The above study concludes that covalent attachment of α-amylase to cellulose dialdehyde coated magnetite nanoparticle results in formation of a novel starch degradation system. The enzyme bearing magnetite particles can be removed easily after the degradation is complete. The degradation efficiency can be controlled by varying amount of cellulose coated, concentration of periodic acid and magnetite content. The proposed magnetic particles have potential to be used in starch degradation based industries.

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