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Research Article

Immobilization of Amylases via Adsorption on Agar-Coated Magnetic Nanoparticles

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Abstract

In this study, a new method for simultaneous synthesis and coating of magnetic iron oxide nanoparticles was employed, where ferric and ferrous ions were co-precipitated within an aqueous solution containing agar at room temperature under inert atmosphere. X-ray diffraction (XRD) analysis indicated that synthesized nanoparticles were pure Fe_3O_4 with a cubic structure and crystallite size ranging between 9.38 and 10.11 nm. The scanning electron microscope (SEM) images demonstrated the increasing surface roughness as concentration of surface coating material increased. The produced magnetic particles were used as a support for α−amylase immobilization by adsorption method. Effects of sonication, immobilization time (0.5; 1; 2; 4; 8; 16 h) concentration of surface coating material (0; 0.5; 1% w/v agar), immobilization pH (pH 3,4,5,6,7) on protein loading, enzyme activity and specific activity were investigated. Sonication did not enhance amylase immobilization. Based on the specific activity of the enzyme, the optimum adsorption was achieved at pH 4 and 5 after 4h-immobilization time, where, compared to the free α−amylase, a 3-fold increase in specific activity and 360% increase in relative activity was measured, respectively.

Keywords: Magnetic nanoparticles, Fungal alpha amylase, Enzyme immobilization, Adsorption method.

Agar-Kaplı Manyetik Nanopartiküller Üzerine Adsorpsiyon ile Amilaz İmmobilizasyonu

Öz

Bu çalışmada, inert atmosfer altında oda sıcaklığında agar içeren sulu bir çözelti içinde ferrik ve ferröz iyonlarının birlikte çökeltildiği, manyetik demir oksit nanopartiküllerin eşzamanlı sentezi ve kaplanması için yeni bir yöntem kullanıldı. X-ışını kırınımı (XRD) analizi, sentezlenen nanopartiküllerin kübik yapıya ve 9.38 ile 10.11 nm arasında değişen kristalit boyutuna sahip saf Fe_3O_4 olduğunu gösterdi. Taramalı elektron mikroskobu (SEM) görüntüleri, yüzey kaplama malzemesi konsantrasyonu arttıkça artan yüzey pürüzlülüğünü gösterdi. Üretilen manyetik partiküller adsorpsiyon yöntemiyle α-amilaz immobilizasyonu için destek olarak kullanıldı. Sonikasyon, immobilizasyon süresi (0,5; 1; 2; 4; 8; 16 saat), yüzey kaplama malzemesi konsantrasyonu (0; 0,5; %1 w/v agar), immobilizasyon pH'ının (pH 3,4,5,6, 7) protein yüklemesi, enzim aktivitesi ve spesifik aktivite üzerine etkileri araştırıldı. Sonikasyon, amilaz immobilizasyonunu arttırmadı. Enzimin spesifik aktivitesine dayalı olarak, optimum adsorpsiyon pH 4 ve 5'te 4 saatlik immobilizasyon süresinde elde edildi, bu koşullarda serbest α−amilaza kıyasla spesifik aktivitede 3 kat artış ve bağıl aktivitede %360 artış ölçüldü.

Anahtar Kelimeler: Manyetik nanopartikül, Fungal alfa amilaz, Enzim immobilizasyonu, Adsorpsiyon metodu.

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1. Introduction

Enzymes, are proteins that catalyze the biochemical reactions in living organisms. Due to their high catalytic activity, selectivity, specificity, and biodegradability, they are often employed in industrial processes. In addition, they can operate at mild pH, temperature, and pressure, which decrease the by-product toxicity and corrosion of process equipment (Schmid et.al., 2001; Koeller & Wong, 2001). However, the recovery of theses biocatalysts from the reaction effluent is challenging and usually they cannot be reused after the first run, which makes their utilization no longer economic (Datta et.al., 2012). This drawback can be overcome by enclosing enzymes via immobilization techniques.

The immobilization allows the easy recovery of enzyme, rapid termination of the chemical reaction and repeated use of the catalyst, which reduce the process cost. In addition, after immobilization, the storage stability, pH, and thermal resistance of the enzymes are usually improved. Based on these advantages, immobilized enzymes have been extensively applied in various applications such as pharmaceutical, food and feed, textile industries, wastewater treatment, medical diagnostics etc.

The commonly employed immobilization techniques involve physical adsorption (Junior et.al. 2016), covalent bonding (Zhu et.al., 2014), crosslinking (Sahutoglu & Akgul, 2015) and entrapment (Bilal & Asgher, 2015) of the biocatalysts. In addition to the immobilization method, the properties of the selected support material also significantly contribute to the efficiency of the biocatalyst.

After the enzymatic processes, the effective separation of immobilized biocatalysts from the reaction mixture is essential for the multiple use of the catalyst. In that respect, coupling enzymes with a magnetic support provides a simple separation of the biocatalytic system by the use of an external magnetic field. Magnetic nanoparticles (MNPs) constitute a promising carrier for the biocatalysts due to their high surface area to volume ratio. Moreover, the presence of hydroxyl groups on their surface facilitates the modification and activation of the particles to ensure enhanced immobilization of the biomolecules. Besides, the high mechanical stability and low porosity of the support provides the spatial orientation and mobility to the protein backbone, essential for the catalytic activity of the enzyme (Li et.al., 2013). All these features led to a substantial improvement in loading capacity of enzymes or biomolecules. More importantly, the magnetic properties of the support provide simple, easy, and cheap separation of the immobilized enzyme from the catalytic medium, ensuring immediate termination of the reaction as well as efficient recovery of the catalyst (Liu et.al., 2018).

In this study, bare and agar coated (0.5%- and 1%- w/v) magnetic nanoparticles were synthesized by co-precipitation and simultaneous-coating synthesis method at room temperature under an inert atmosphere. Synthesized particles were characterized by X-ray diffraction (XRD) and scanning electron microscope (SEM). Then, α−amylase was immobilized onto particles with adsorption method. Immobilization parameters such as immobilization pH, time, sonication, and surface coating material were investigated.

2. Materials and Methods

Iron (II) chloride tetrahydrate ($FeCl_2$. $4H_2O$), iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) and sodium hydroxide (NaOH), carboxymethylcellulose sodium salt (NaCMC), pectin and agar-agar were purchased from Carlo Erba, AFG Bioscience, Fisher Scientific, respectively, and used as obtained without further purification. Milli-Q water (15.0 MΩcm at 21.8°C) was used for all steps of synthesis that required water.

2.1. Synthesis of Magnetic Nanoparticles

Magnetic nanoparticles were synthesized by co-precipitation method, where 0.25 M $FeCl_2$.4H2O and 0.5 M $FeCl_3.6H_2O$ aqueous solutions were prepared. Then, equal volumes of prepared ferric and ferrous solutions were mixed. 2M NaOH solution was used as a precipitation agent and added dropwise to the mixture under continuous stirring at room temperature until the solution became completely black. The reaction mixture was further deoxygenated by the flow of nitrogen gas. After the reaction, solution was repeatedly centrifuged at 3000 rpm for 3 minutes and washed until the supernatant pH was neutral. Precipitate was collected with an external magnetic field and dried in an oven at 40ºC under vacuum pressure.

Polysaccharide-coated magnetic nanoparticles were synthesized by the simultaneous synthesis-coating method in the media containing agar-agar at different concentrations (0.5% and 1% w/v).

Synthesized magnetic nanoparticles were characterized by X-ray diffraction (XRD) and scanning electron microscope (SEM, HITACHI TM3030Plus Benchtop).

2.2. Immobilization Studies

Fungal α−amylase was immobilized onto synthesized bare and 0.5% and 1% w/v agar coated magnetic nanoparticles by adsorption method. In the immobilization studies effects of parameters such as sonication, immobilization time (0.5; 1; 2; 4; 8; 16 h) concentration of surface coating material (0; 0.5; 1% w/v agar), immobilization pH (pH 3,4,5,6,7) on the amount of bonded protein, enzyme activity and specific activity were investigated. The protein content of the solutions was measured using Bradford method (Bradfprd, 1976), enzyme activity was estimated with starch-iodine assay (Xiao et.al., 2006).

3. Results and Discussion

3.1. Characterization of Magnetic Nanoparticles

The crystallinity and structure of synthesized particles were identified by powder X-ray diffraction (XRD) on Rigaku Miniflex 600. All XRD patterns were collected using a Cu Kα radiation source (λ = 1.54 Å), in a measuring range of 10- 80º on 2θ scale at a scan speed 0.02º s^{-1}.

Figure 1 illustrates the diffraction pattern of synthesized bare and coated iron oxide nanoparticles. The crystalline structures of particles were identified by matching specific peak positions and relative intensities to JCPDS (Joint Committee on Powder Diffraction Standards) card, file No. 00-019-0629. The characteristic diffraction peaks at 2θ = 30.54, 36.00, 43.54, 53.82, 57.66, and 63.28, and their indices (2 2 0), (3 1 1), (4 0 0), (4 2 2), (5 1 1), and (4 4 0), respectively, coincided with the peaks in the database for Fe_3O_4. This revealed that resultant nanoparticles were

pure Fe_3O_4 with a cubic structure. The same peaks appeared for bare (Fig.1-c) and coated (Fig.1-a&b) magnetic particles, indicating that no other phase, such as $Fe(OH)_3$ or Fe_2O_3 -the usual products in a chemical co-precipitation procedure, was formed. Thus, the crystalline structure of Fe_3O_4 did not change on modification of particles with agar and agar coating occurred only at the surface of Fe_3O_4.

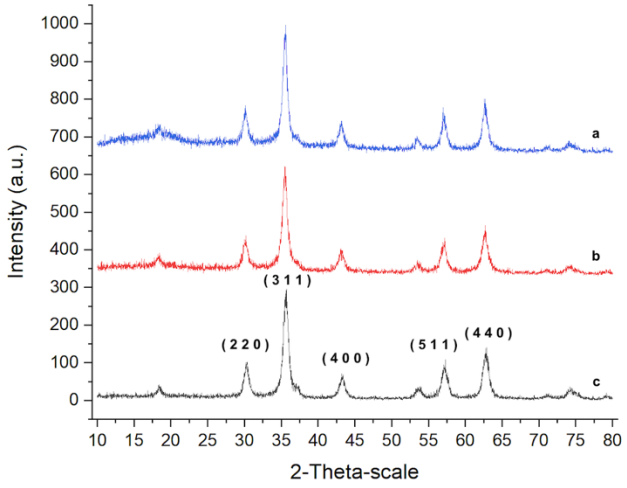

Fig. 1 XRD results of synthesized magnetic nanoparticles (a) 1% agar-coated, (b) 0.5% agar-coated, (c) naked particles

Additionally, average crystallite sizes of synthesized magnetic particles were calculated using Debye-Scherrer Equation (Equation 1):

$$D_{nm} = \frac{K\lambda}{\beta cos\theta} \qquad (1)$$

where K is constant, λ is X-ray wavelength and β is the peak width of half-maximum.

Crystallite size of bare magnetic particles, 0.5% agar-coated magnetic particles and 1% agar-coated magnetic particles found as 9.55 nm, 9.38 nm, and 10.11 nm, respectively.

The magnetic behavior of the agar-coated iron oxide nanoparticles synthesized in 0.5% (w/v) aqueous agar solution is also shown in Figure 2, where the response of the particles to an external magnet is demonstrated. The homogeneously dispersed solution (Fig.2-a) having dark brown/black color, immediately started to decolorize indicating the migration of the particles towards the magnet (Fig. 2-b). After 71 s the aqueous solution containing magnetic nanoparticles was completely cleared, gathering all the particles to the magnet (Fig.2-c).

Fig. 2. Synthesized 0.5% w/v agar coated magnetic particles with an external magnetic field after (a) 0 s, (b) 15 s and (c) 71s

Scanning electron microscopy (SEM) was applied to evaluate the surface morphology of the synthesized magnetic particles (Fig.3). The bare magnetic particles had smooth surfaces and sharp edges (Fig.3-a), with the addition of agar in synthesis media, the distinctive surfaces and edges smoothened (Fig.3-b) and eventually the whole surface was roughened up (Fig. 3-c). This showed that agar-coating increased the roughness, i.e., the surface area of the magnetic support.

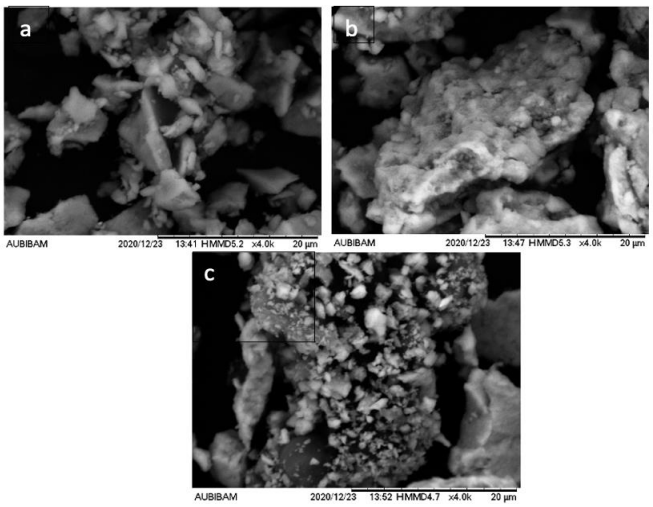

Fig. 3. SEM images (magnification: ×4.0K) (a) bare, (b) 0.5% w/v agar-coated and (c) 1% w/v agar-coated particles

3.2. Immobilization Studies

1 mg/mL fungal α-amylase was immobilized onto 0.01 g bare and 0.5% w/v agar-coated magnetic particles. Particles were suspended in enzyme solution at pH=7, and the immobilization was carried out in an ultrasonic bath (40kHz) and at room conditions for 30 min. Then, particles were washed 2 times and the amount of bonded protein was calculated by subtracting the amount of protein in the wash solutions from the total protein in the initial enzyme solution before immobilization.

Table 1. Effect of sonication in enzyme immobilization

Immob. method	pH	Particle	Bonded Protein (%)
Room Cond	7	Free Enzyme	100 (0.09*)
		Bare	53.10
		0.5% Agar-Coated	65.61
Sonication	7	Free Enzyme	100 (0.12*)
		Bare	53.95
		0.5% Agar-Coated	44.72

**protein amount in free enzyme (mg protein)*

No evidence, suggesting that sonication enhanced enzyme immobilization, was observed. On the contrary, exposure to sonication resulted in a change in color of washing solutions (Fig 4). Thus, Bradford's analysis did not work properly. After that, all immobilization studies were performed at room conditions.

To investigate the effect of immobilization pH, studies were performed with enzyme solutions at different pH (0.05 M pH 3-4-5-6-7 citric acid-phosphate buffers). Amount of bonded protein

was calculated, and enzyme activity and specific activity at pH 4.5 and 55ºC were measured by starch-iodine method.

a) b)

Fig.4. Washing solutions of exposure the sonication (right) and room conditions (left), (a) bare and (b) 0.5%w/v agar-coated particles

When the immobilization pH was 3, 99.89% of the enzyme was adsorbed on the magnetic support (Table 2). However, the measured enzyme activity was very low, specifying that the bound enzymes did not work properly. This might indicate that the three-dimensional conformation of the enzyme exposed the functional groups on the protein backbone that enhanced the adsorption of the enzyme to the support. However, the unfavorable positioning of the enzymes in the immobilization process probably hindered the active sites and reduced their activity.

Table 2. Effect of pH in enzyme immobilization

pH	Enzyme	Bonded Protein (%)	Act (U)	Sp. Act. (U/mg prot.)	Relative Act. (%)
3	Free	100 (0.11*)	0.56	4.95	100
	Immob.	99.89	0.45	4.00	80.81
4	Free	100 (0.07*)	14.7	221.7	100
	Immob.	20.92	0.57	40.7	18.36
5	Free	100 (0.09*)	19.4	223.1	100
	Immob.	3.74	0.49	152.2	68.24
6	Free	100 (0.06*)	18.1	291.3	100
	Immob.	18.13	0.45	40.0	13.71
7	Free	100 (0.12*)	24.16	198.4	100
	Immob.	26.21	0.59	18.45	9.30

**protein amount in free enzyme (mg protein)*

Since the main purpose of enzyme immobilization is to use enzymes repeatedly and in the most efficient way, it is more essential to obtain magnetic particles with high specific activity rather than the high amount of protein loading. Therefore, when the specific activities were evaluated, it was seen that the immobilization processes at pH 4 and 5 gave more efficient results, and the study was continued in environments with these pH values.

The studies on the effect of immobilization time were conducted for periods of 0.5, 1, 2, 4, 8, and 16 hours, using pH 4 and pH 5 buffer solutions. The 0h results in Table 3 represent the free enzyme activity values. In general, the prolonged immobilization time resulted in increased enzyme loading. This, in turn, led to a substantial decrease in enzyme activity. It was considered that the high amylase loading resulted in an intermolecular steric hindrance, which restrained the mobility of the protein backbone and limiting the catalytic activity of the enzyme, as well as diminishing the diffusion of the substrate restricting it from reaching the active site of the enzyme. It was concluded that excessive loading should be avoided for optimum catalytic performance (Bai et.al, 2011; Sohrabi et.al., 2013; Jiang et.al., 2016; Ranjbakhsh et.al., 2012).

Table 3. Effect of time in enzyme immobilization

t (h)	pH	Bonded Protein (%)	Act (U)	Sp. Act. (U/mg prot.)	Relative Act. (%)
0	4	100 (0.067*)	6.43	96.1	100
	5	100 (0.078*)	11.04	141.5	100
0.5	4	17.078	0.482	42.1	43.85
	5	7.612	0.422	71.1	73.94
1	4	4.353	0.370	127.1	131.8
	5	6.158	0.363	81.7	57.77
2	4	3.907	0.424	162.2	168.7
	5	4.167	0.375	115.4	120.1
4	4	10.716	0.348	48.5	50.49
	5	0.962	0.382	509.3	360.0
8	4	57.149	0.098	2.6	2.67
	5	2.725	0.084	39.3	27.79
16	4	72.218	0.125	2.6	2.68
	5	13.223	0.074	7.2	5.06

**protein amount in free enzyme (mg protein*

The specific activity reached a maximum value when immobilization pH was adjusted to 5 and time was restricted to 4 h (Fig 6.). Although, the absolute activity of the immobilized amylases was relatively low (0.382 U) compared to other measured values (0.482 U at pH=4 for 0.5 h immobilization time), the specific activity attained a maximum of 509.3 U/mg protein. The results demonstrated that for a considerably small amount of enzyme a substantial activity was accomplished. Moreover, compared to the free enzyme, where no limitations in terms of enzyme conformation and substrate/product diffusion is experienced, the immobilized amylase exhibited an outstanding catalytic performance of 360% relative activity. This implied that the immobilization process, despite its restrictions, stabilized and enhanced the catalytic activity of the enzyme.

4. Conclusions and Recommendations

The present study aimed to investigate the effect of immobilization parameters such as sonication, immobilization time and immobilization pH on bonded protein amount, enzyme activity and specific activity of amylases. A facile method for simultaneous synthesis and coating of the magnetic support was applied. The functional groups on the synthesized magnetic nanoparticles were agar. The XRD patterns proved that pure Fe_3O_4 with crystallite size of approximately 10 nm was produced and the coating process did not affect the magnetite's structure. An immobilized process at room conditions at pH=5 for 4 h

resulted in the best α-amylase performance, which showed a specific activity increase of 360% compared to the free enzyme.

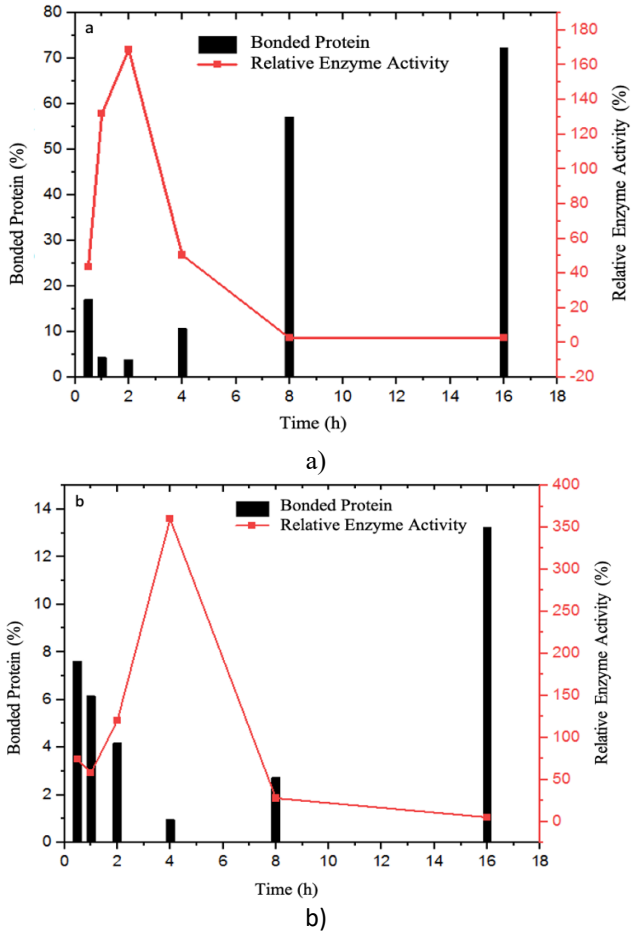

Fig.6. Effect of immobilization time on the amount of bonded protein and relative activity of enzymes adsorbed on 0.5% w/v agar-coated magnetic particles at (a) pH 4 and (b) pH 5.

Experiments showed that alpha amylase was adsorbed onto synthesized magnetic nanoparticles successfully and worked properly. However, leakage of the immobilized enzyme was also observed, indicating that future studies should be focused on methods providing firmer attachment of the enzymes to the magnetic support.

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