Immobilization of Pectinase Enzyme on Hydrophilic Silica Aerogel and Its Magnetic Nanocomposite

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ABSTRACT: *In this work, Aspergillus niger pectinase was immobilized on hydrophilic silica aerogel and its magnetic nanocomposite by adsorption method, and the performance of these supports in pectinase immobilization was compared. Physical and chemical properties of supports and the immobilized pectinase were characterized by Brunauer–Emmett–Teller (BET) analysis, Field Emission Scanning Electron Microscope (FESEM), Fourier Transforms InfraRed (FT-IR) spectroscopy, and Vibrating Sample Magnetometer (VSM). The results showed that the pectinase was successfully immobilized onto both supports. The kinetics of the immobilized pectinase followed Michaelis–Menten model. The maximum reaction rate (V_{max}) and affinity of immobilized pectinase to the substrate (K_m) in pure silica aerogel were higher than in magnetic silica aerogel. The maximum monolayer adsorption capacity of the pure silica aerogel (q_{max}=129.17 mg/g) was higher than magnetic silica aerogel (q_{max}=53.42 mg/g) based on Langmuir isotherm. The thermal stability of the immobilized pectinase was improved toward free pectinase. The reusability tests of immobilized pectinase showed that magnetic silica aerogel had better operational stability than pure silica aerogel because of higher mechanical resistance and retained 57% of its initial activity after 10 repetitive cycles.*

KEYWORDS: *Adsorption; Biocatalysis; Immobilization; Pectinase; Silica aerogel.*

INTRODUCTION

Pectinases, as a large group of enzymes, break down pectin into simpler molecules such as galacturonic acid and are widely produced by many microorganisms. These enzymes have wide applications in the fruit juice and wine industry for clarifying [1]. Despite the very useful properties of these enzymes, like other enzymes, industrial applications of pectinases are limited due to their high cost, low stability under processing conditions, and enzyme recovery problems from the reaction mixture. These problems can be overcome by immobilizing pectinase in solid support in an appropriate manner [2, 3]. Considerable effort is still being dedicated to the search for new support

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materials and novel techniques for immobilization. The type of support , as well as the method of immobilization, influences the activity and operational stability of immobilized enzymes [4]. There are various techniques to enhance enzyme stability, including entrapment, adsorption, covalent binding, and intermolecular cross-linking of enzyme molecules [1,5]. Among immobilization methods, adsorption is the most economically and industrially favorable option. In this method, the weak forces between a support and the enzymes include hydrogen bonding, van der Waals forces, and hydrophobic interactions [6-8].

Different supports, for example, alginate beads [1], alginate-coated chitin [2], chitosan-coated chitin [3], chitosan-magnetic particles [9], activated alginate-montmorillonite beads [10], activated montmorillonite [11], thereusable polymer [12], polyacrylonitrile copolymer membrane [13], celite [14], polysulfone membranes [15], cobalt ferrite particles [16], oxidized pulp fiber [17], agar-agar [18], silica-based supports [19], silica-coated chitosan particle [20], and porous hydroxyapatite/calcium alginate composite beads [21] have been used for the immobilization of pectinase. Appropriate support selected for immobilization would have high porosity, large surface area, high capacity (or affinity), long life, and high chemical and thermal resistance [6]. Silica aerogels contain all the mentioned characteristics as well as being mesoporous and having a low mean free path of diffusion, which is suitable for application as catalyst and biocatalyst supports. Almost all aerogels are derived from gels made through sol-gel chemistry. If a gel is dried in the supercritical condition or ambient condition after appropriate modification, the structure of the gel is maintained and aerogel is formed [22]. In recent years, silica aerogels have been used as enzyme immobilization support by encapsulation and adsorption methods. All these used supports were dried in supercritical condition which is a dangerous and costly method compared to the ambient pressure drying method [23-25]. The main purpose of enzyme immobilization is to reduce process costs. Thus the use of expensive support cannot be economically justified [26].

In this work, pure silica aerogels and their magnetic nanocomposites were synthesized by a cheap and safe ambient pressure drying method, and their performance was studied as support in *Aspergillus niger* pectinase immobilization by adsorption, and the immobilization efficiency was investigated. As mentioned above in some previous research, silica-based supports were used for pectinase enzyme immobilization via covalent attachment [19, 27]. Silica aerogels have a much higher specific surface area and porosity than other silica supports, which makes them have a much higher capacity for theadsorption of enzymes. Also, the presence of porosity and open pores on the surface of silica aerogel causes the enzyme to be trapped. To our knowledge, silica aerogel has never been used as a support material for pectinase immobilization by the adsorption method. In the adsorption immobilization method, the native structure of the enzyme doesn't change.

EXPERIMENTAL SECTION

Materials

Pectinase from *Aspergillus niger* and polygalacturonic acid were purchased from Sigma Aldrich (Saint Louis, MO, USA). 3,5-dinitrosalicylic acid (DNS), ammonium solution (NH3, 25%), and sodium acetate were purchased from Merck Chemicals Co. (Darmstadt, Germany).

For the preparation of silica aerogels, sodium silicate (water glass) with 1.35 specific gravity, ammonium hydroxide, n-Hexane, isopropyl alcohol (IPA), and Hexamethyldisilazane (HMDZ) from Merck Company (Darmstadt, Germany) were prepared. For ion exchange of sodium silicate, Amberlite IR-120 H^+ was also provided by Merck Company. Iron oxide particles were prepared by the US Research Nanomaterials Company. All the other chemicals used were of analytical grade.

Preparation of silica aerogel and magnetic silica aerogel

Silica aerogel and its magnetic nanocomposite were used for theimmobilization of pectinase by the adsorption method. Both supports were synthesized by the ambient pressure drying method. For synthesis support, initially, the sodium silicate solution was diluted with deionized water by 1:4 volumes. In the next step, the removal of unwanted Na^+ ions from the sodium silicate solution was carried out by mixing the diluted sodium silicate solution with the ion exchange resin in equal volume proportion. After the formation of silicic acid with a pH of around 2, ammonium hydroxide solution (1.0 M) was added to raise its pH to 4 for gelation. The obtained silica sol was transferred to the Teflon vessels immediately and a hydrogel formed. The gel was kept in the oven at 50°C

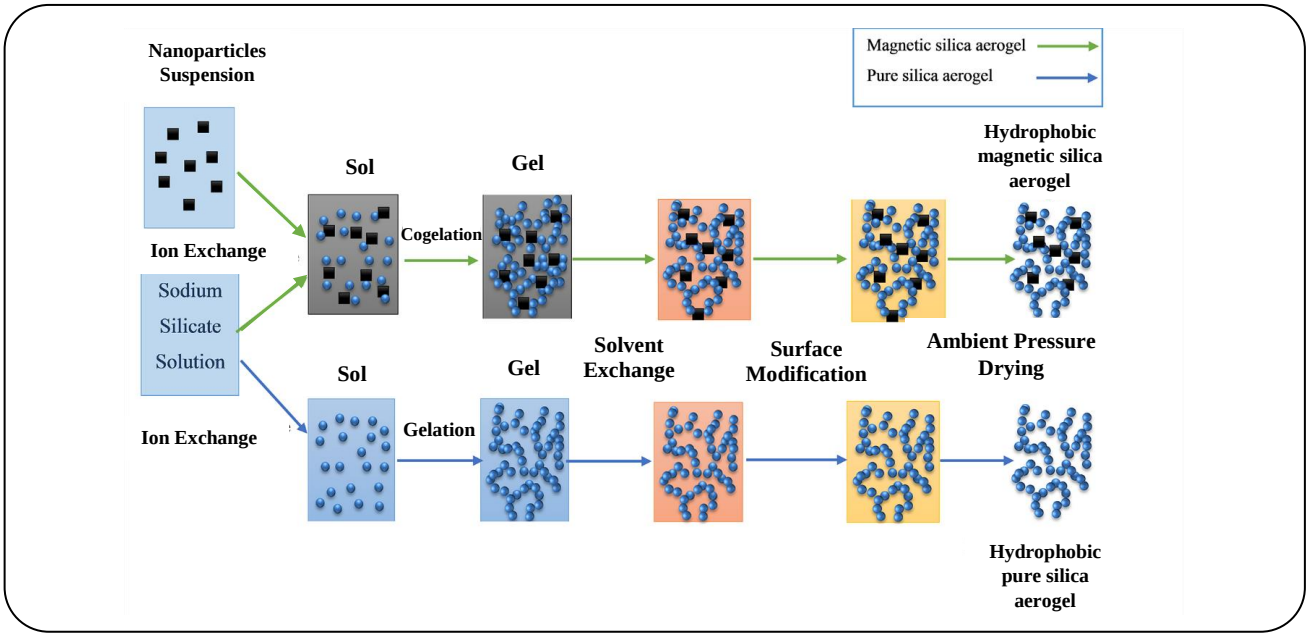

Fig. 1: Schematic representation of the synthetic procedure of pure and magnetic silica aerogels.

for 180 min to strengthen the silica network. The water present in the pores of the gel was exchanged with isopropyl alcohol and normal hexane (1:1 v/v) by placing the gel in the oven for 18 h at 50°C. After solvent exchanging and aging, the wet gels were immersed in HMDZ/n-hexane (1:4 v/v) solutions for 12 h at 50°C to modify the surface. Finally, the modified gels were dried at room temperature for 24 h and then at 50, 80, and 120°C for 2 h, respectively. In thesynthesis ofhydrophobic magnetic silica aerogel, in the first step, 0.5 g iron oxide particles were dispersed in 20 cc ofdeionized water by an ultrasonic mixer. The power of the mixer was 70 watt, and solutions were mixed for 1 hr. Then water glass was added to the mixture in 1 to 4 ratios. The other steps were done as mentioned above [7]. The resulting supports had superhydrophobic properties. After heating the aerogel and its composite at 500℃ for 2 h, the -CH_3 groups changed to -OH groups and the supports became hydrophilic [6]. Fig. 1 shows the schematic representation of the synthetic procedure of pure and magnetic silica aerogels.

Pectinase immobilization

For immobilization of pectinase, 10 mL of pectinase solution (10 mg/mL in 50 mM, pH 5.0 sodium acetate buffer) was mixed with 0.1 g of supports for 90 min to complete the adsorption process. The immobilization was conducted at ambient temperature, astirring rate of 500 rpm. After that, the suspension was filtrated and the supports with immobilized pectinase were separated, washed with phosphate buffer to remove the unabsorbed enzyme, and dried in the air. The filtrate and washing solutions were collected for protein determination [7]. Fig. 2 shows the schematic representation of the adsorption of pectinase on pure and magnetic silica aerogels.

TheKinetics of the adsorption was studied for 150 min using sodium acetate buffer with a pH of 5 and in the same conditions as mentioned above.

Characterization of supports and immobilized pectinase

The specific surface area was determined by Brunauer-Emmett-Teller (BET) method (BEL Sorp-II mini, BEL Japan Inc., Osaka, Japan). FT- IR spectra of the supports and immobilized pectinase were obtained with a Fourier transform infrared spectroscopy (FTIR, PU 9800, from Philips, Netherlands) in the range of 4000-400 cm^{-1}. The pore structure and particle morphology were characterized by field emission scanning electron microscopy (FESEM, Mira 3-XMU, Tescan USA Inc.)

Pectinase activity assay

Pectinase activity was determined by estimating the amount of reducing sugar released under assay conditions using polygalacturonic acid as a substrate. One unit of activity was defined as the amount of enzyme required to

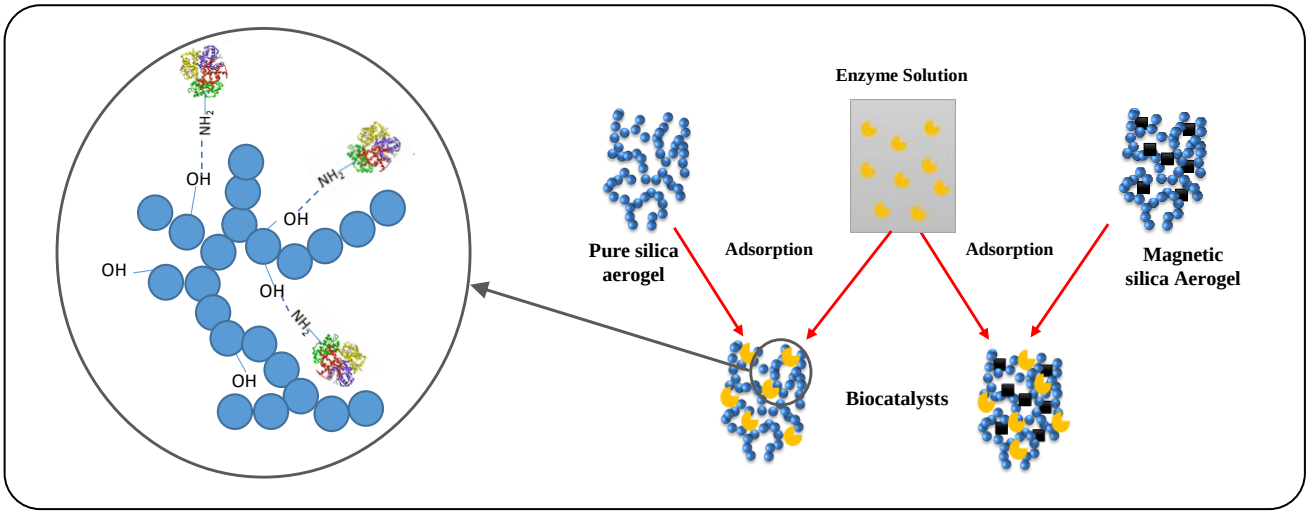

Fig. 2: Schematic representation of the synthesis of biocatalysts

release 1 µmol of galacturonic acid per mL per minute under standard assay conditions. The enzymatic activity of pectinase was determined by adding 100 µL of enzyme solution or suspension containing the same amount of enzyme protein to 400 µL of 0.5 % (w/v) poly galacturonic acids in 20 mM sodium acetate buffer, pH 5. After 30 min at 37°C, the reaction was stopped by adding 3,5-dinitrosalicylic acid, and the reducing sugars were spectrophotometrically measured at 540 nm [3]. The protein concentration was determined using bovine serum albumin as standard by the Lowry method [28]. All experiments were carried out in triplicate under identical conditions.

Immobilization yield

Immobilization yield percentage (%) was calculated as in the following equation (1):

$$\%\ \text{Immobilization yield} = \frac{\text{Amount of protein loaded}}{\text{Amount of protein introduced}} \times 100 \quad (1)$$

Where the amount of protein loaded was calculated as the difference between the initial protein concentration and the protein concentration in the fluid remaining after enzyme immobilization [6].

Determination of the pectinase kinetic parameters

Michaelis-Menten (M-M) model (Eq. 2) was used to describe the dependence of enzyme activity on substrate concentration for immobilized pectinase on hydrophilic silica aerogel and its magnetic composite.

$$v = \frac{v_{max} S}{K_m + S} \quad (2)$$

The apparent M-M constant (K_m) and the highest reaction rate (v_{max}) were determined by applying the activity assay (v) for different pectin concentrations (S) ranging from 0 to 30 mg/mL under the assay conditions [6].

Analysis of adsorption isotherm of pectinase on silica aerogel and its nanocomposite

To determine the adsorption capacity of hydrophilic silica aerogel and its magnetic composite for pectinase, the Langmuir and Freundlich adsorption isotherms were employed [29]. Langmuir isotherm corresponds to the immobilization in a monolayer, and Freundlich isotherm corresponds to multilayer immobilization [6].

The Langmuir isotherm is typically represented by Eq. 3:

$$q = \frac{q_{max} C_e}{K_d + C_e} \quad (2)$$

Where q is the adsorption capacity defined as the amount of protein adsorbed per unit weight of supports (mg/g), C_e is the residual amount of protein in the unit volume of liquid phase (mg/mL), q_{max} is the maximum adsorption capacity (mg/g) and K_d is the Langmuir constant (mg/mL) related to the energy of adsorption [6].

The Freundlich formula is described by Eq. (4):

$$q = K_F C_e^{\frac{1}{n}} \quad (3)$$

Where K_F (mL/g) and 1/n (unitless) are the Freundlich constants. K_F indicates the support capacity, and 1/n is the heterogeneity factor [29].

To determine the adsorption isotherm, different concentrations (0.2-3 mg/mL) of pectinase were incubated with 10 mg/mL of supports at ambient temperature and pH of 5 for 90 min to reach equilibrium.

Thermal stability

The thermal stability tests were carried out when reaction mixtures containing free or immobilized enzymes were kept at 70˚C for various periods (30-120 min). At designated time intervals, the relative activity of each sample was determined as described above. The relative activity was defined as the ratio of the residual activity to the initial activity.

Reusability assay

To test the reusability of immobilized pectinase, the supports were separated from the reaction medium. Post-treatment of biocatalysts was done by washing three times with sodium acetate buffer (pH 5.0) and drying for 1 hour. Recovered immobilized pectinase was used in the next activity assay with fresh substrates. The assay condition was the same as described above.

RESULTS AND DISCUSSION

Physiochemical properties of supports and immobilized pectinase

Fig. 3 (a, b, c, and d) presents FESEM features of silica aerogel and magnetic silica aerogel before and after the immobilization of pectinase. Since the coagulation method was used in magnetic silica aerogel synthesis, the presence of iron oxide nanoparticles in the first step of synthesis in the gel cavities and the formation of some Fe-O-Si bands leads to a reduction in surface modification. As a result, the specific surface area and the mean pore size of nanocomposite supports were decreased compared to pure silica aerogel supports. After pectinase immobilization (Fig. 3b and d), it was observed that the surface morphology of supports was changed and the particles seem to be aggregated, which could be attributed to the creation of some bonds after pectinase adsorption. On the other hand, after immobilization, a layer of the enzyme may cover the surface of supports and decrease the porosity.

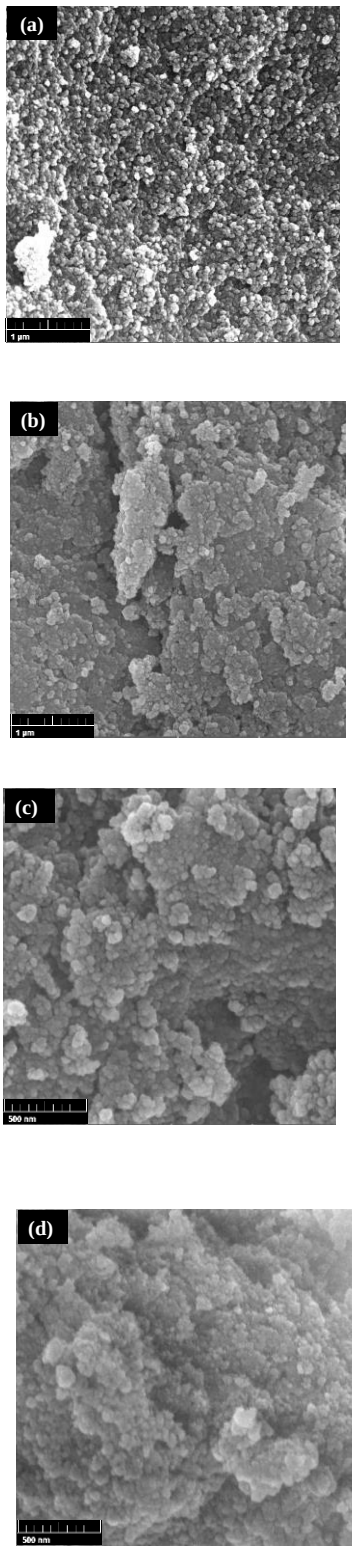

Fig. 3: FESEM micrographs of (a) silica aerogel; (b) silica aerogel-pectinase; (c) magnetic silica aerogel and (d) magnetic silica aerogel-pectinase

Table 1: Specific surface area and mean pore size of the supports with and without pectinase immobilization.

Materials	Surface area (m^2/g)	Mean pore size (nm)
Pure silica aerogel	579	16.45
Pure silica aerogel- pectinase	533	12.24
Magnetic silica aerogel	499	8.35
Magnetic silica aerogel- pectinase	485	8.24

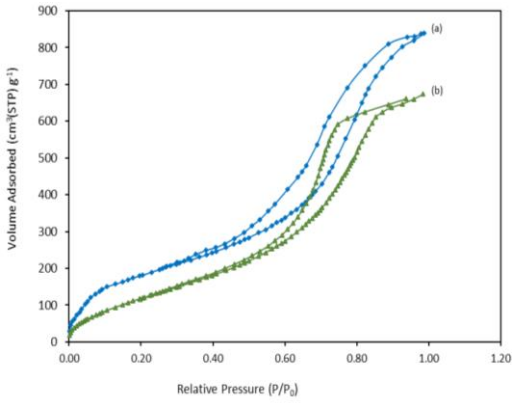

Fig. 4: N_2 adsorption- desorption isotherms of (a) pure silica aerogel (b) magnetic silica aerogel.

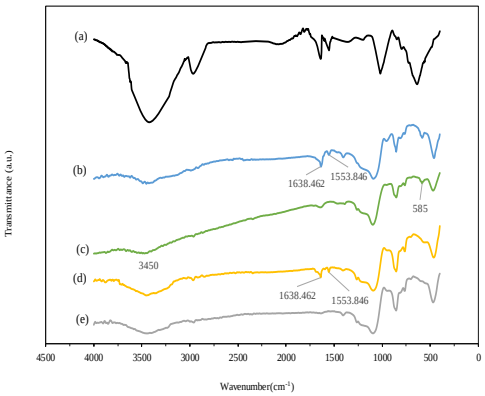

Fig. 5: FT-IR spectra of the supports before and after immobilization: (a) free pectinase (b) magnetic silica aerogel-pectinase, (c) magnetic silica aerogel (d) silica aerogel-pectinase (e) pure silica aerogel.

The BET analysis confirmed these results. Specific surface area and mean pore size in both supports were decreased after enzyme immobilization (Table 1). Fig. 4 shows the nitrogen adsorption-desorption isotherms of supports, which exhibited a type IV isotherm with hysteresis loop H1.

FT-IR analysis was used to confirm the adsorption of pectinase on silica aerogel and magnetic silica aerogel. Fig. 5 (a, b, c, d and e) shows the FTIR spectra of the free pectinase and the supports before and after immobilization. The pectinase enzyme has two characteristic bands at 1638.46 and 1553.8 cm^{-1} (primary and secondary amino groups). These peaks in both supports are visible after pectinase immobilization (Fig. 5 b and d). This indicates the adsorption of pectinase onto supports. In all samples, there was a major peak at around 3450 cm^{-1} which indicated the existence of O-H groups. On the other hand, in magnetic silica aerogel before and after immobilization, there was a characteristic peak at 585cm^{-1} indicating the existence of a Fe–O bond that confirmed the presence of Fe_3O_4 particles.

The magnetization curve of synthesized nanocomposites at room temperature (298 K) was obtained by the use of a Vibrating Sample Magnetometer (VSM) with a maximum magnetic field of 10 kOe (Fig. 6). The results indicated that the nanocomposite exhibited ferromagnetic behavior and the value of saturation magnetization (Ms) was 4.66 emu/g.

Effect of reaction time on pectinase immobilization efficiency

The effect of adsorption time on the pectinase immobilization yield is shown in Fig. 7. According to this figure, the adsorption process was completed at a reaction time of 90 min for both supports. Thus the reaction time of 90 min was used to immobilize the pectinase. These results demonstrated that the adsorption of protein onto inorganic supports is a fast process [16]. Immobilization yield for pure silica aerogel at all times was higher than magnetic silica aerogel. As shown in the previous section, the porosity of pure silica aerogel was higher than magnetic

silica aerogel, so in the pure silica aerogel, the capacity and affinity to pectinase molecules were higher too.

Kinetic parameters of the immobilized pectinase

Kinetic studies show that the dependence of silica aerogel and magnetic silica aerogel immobilized pectinase activity on substrate concentration can be described by the M–M model (Fig. 8). The co-relation coefficients (R^2) of the curves were 0.98 and 0.99 for silica aerogel-pectinase and magnetic silica aerogel-pectinase respectively. Table 2 shows the kinetics constants of free and immobilized pectinase for the substrate. The v_{max} is the intrinsic catalytic character of the enzyme and K_m reflects the affinity between theenzyme and substrate [10].

As shown in Table 2, v_{max} was decreased upon immobilization from 55.2 µmol/mL.min for the free one to 16.9 µmol/mL.min for silica aerogel-pectinase and 15.76 µmol/mL.min for magnetic silica aerogel-pectinase. These results show that immobilization had an effect on the enzyme kinetics and reduced activity was the result of a structural change induced by electrostatic interactions during the adsorption of a protein and the diffusional restriction of the substrate to the active site of the enzyme [11]. Between immobilized pectinase on two supports, the silica aerogel-pectinase had higher activity. As shown before, pure silica aerogel had higher porosity than magnetic silica aerogel. On the other hand, the porosity in pure silica aerogel is higher than inmagnetic silica aerogel, which would increase the rate of diffusion, substrate, and product to and from the immobilized enzyme, respectively [30]. The higher K_m value indicated a lower affinity of substrate and enzyme. As shown in Table 2 the affinity of immobilized pectinase to its substrate was lower than the free pectinase (higher k_m value), probably due to the conformational changes in the structure of pectinase introduced by the immobilization that led to alower accessibility of the substrate to the active site of the immobilized pectinase. In contrast, the affinity of silica aerogel-pectinase to the substrate was higher (K_m=2.31) than magnetic silica aerogel-pectinase (K_m=3.03) due to the higher porosity of pure silica aerogel. As mentioned before, in magnetic silica aerogel modification of thesurface was lower than inpure silica aerogel. In the modification step the -CH_3 groups were formed. For obtaining hydrophilic silica aerogels and their nanocomposites, the -CH_3 groups that were formed in the modification step, were exchanged

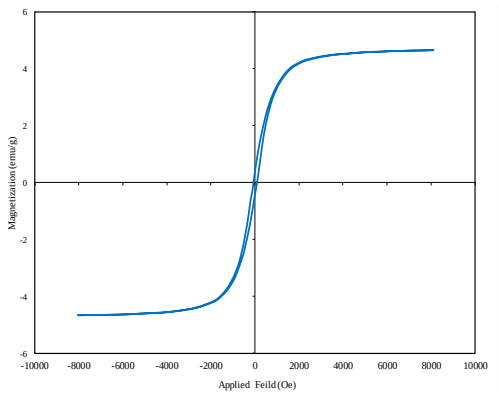

Fig. 6: Room temperature (298K) magnetization curve for magnetic silica aerogel nanocomposite.

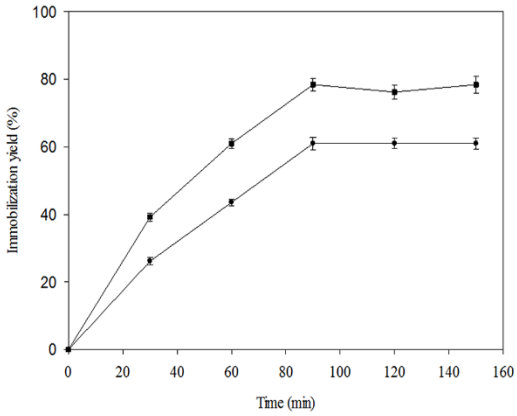

Fig. 7: The effect of immobilization time on the pectinase adsorption onto pure silica aerogel ■ Magnetic silica aerogel ●.

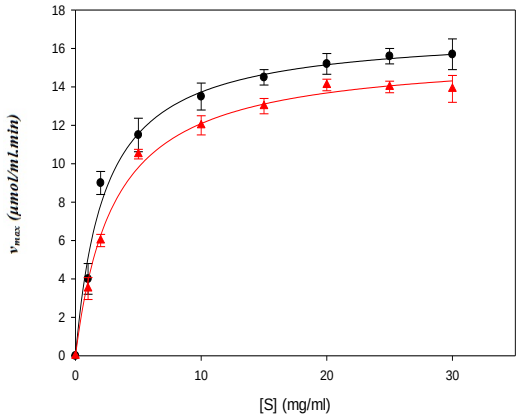

Fig. 8: Michaelis–Menten kinetic curves of pure silica aerogel-pectinase ● Magnetic silica aerogel-pectinase▲.

Table 2: Comparison of kinetics Parameters for Free and Immobilized pectinase.

	K_m (mg/mL)	v_{max} (μmol/mL.min)
Pectinase immobilized on the magnetic silica aerogel	3.03	15.76
Pectinase immobilized on the pure silica aerogel	2.31	16.9
Free pectinase	2.1	55.2

Table 3: Adsorption isotherm parameters of pectinase on pure silica aerogel and magnetic silica aerogel.

Support	Langmuir			Freundlich		
	q_{max} (mg/g)	K_d (mg/mL)	R^2	K_F (mL/g)	1/n	R^2
Pure silica aerogel	129.17	7.63	0.97	15.5	0.75	0.97
Magnetic silica aerogel	53.42	3.42	0.98	12.27	0.61	0.97

with -OH groups in the calcination step. As a result, more hydroxyl groups were found in pure silica aerogel and this material is highly hydrophilic. The presence of many functional hydroxyl groups on the surface of pure silica aerogel enhances the surface attachment of the enzyme, immobilization efficiency, and their affinity to biomolecules [31].

Adsorption isotherm of pectinase on aerogel and magnetic silica aerogel

Fig. 9 shows that the experimental data of pectinase adsorption on silica aerogel and magnetic silica aerogel can be fitted by Langmuir and Freundlich isotherms (Eqs. 3 and 4). Table 3 gives the values of the Langmuir and Freundlich constants and the regression coefficients for the two supports. As shown in this table, for both supports the regression coefficients of the Langmuir model were higher than the Freundlich model, which implied the formation of a monolayer on the support surface. This result indicates an energetically homogeneous surface where all affinities of the binding sites for the enzyme molecule are identical [29,30,32] According to the Langmuir model, the maximum monolayer adsorption capacity of the pure silica aerogel (q_{max}=129.17 mg/g) was higher than magnetic silica aerogel (q_{max}=53.42 mg/g). Also, Table 3 shows the affinity between pure silica aerogel and the pectinase (K_d=7.63 mg/mL) was stronger compared to that of magnetic silica aerogel (K_d=3.42 mg/mL).

The term l/n (Freundlich constant) is the heterogeneity factor of the surface, which for both supports was almost similar and between 0.6 and 0.7. l/n would be closer to 1.0 as surface homogeneity increased. Thus the support surface can be considered homogeneous according to the 1/n values [33].

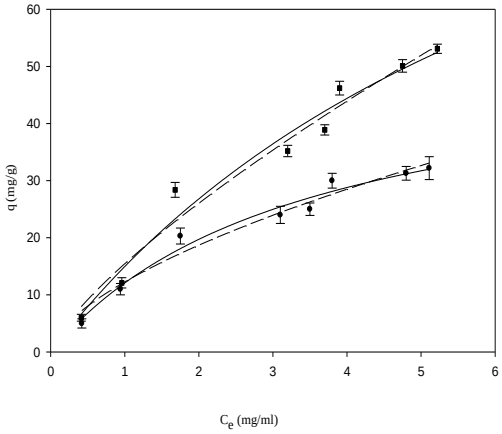

Fig. 9. Adsorption equilibrium of pectinase on pure silica aerogel ■ Magnetic silica aerogel ● at ambient temperature (solid line for Langmuir model, dashed line for Freundlich model).

Thermal stability

Improvement in enzyme stability is one of the reasons for immobilization [11]. The free and immobilized pectinase was incubated at 70˚C and their activities were measured at 30 min intervals for 2 h. The thermal stability of the free and immobilized pectinase was evaluated as shown in Fig. 10. The thermal stability of the immobilized pectinase was improved toward free pectinase. The relative activity was about 75% and 63% for immobilized enzyme on pure silica aerogel and magnetic silica aerogel respectively after 2 h but it reached zero for free pectinase in this time (Fig. 10). The more rigid structure of the immobilized system in contrast to the free enzyme can be the reason for this behavior [6].

Table 4: Comparison between previous works and the present one.

Support type	Immobilization method	Relative activity	Ref.
Alginate-coated chitin	Adsorption	After 9 cycles: 50%	[2]
Activated alginate-montmorillonite beads	Covalent binding	After 6 cycles: 53%	[10]
Activated montmorillonite	Covalent binding	After 6 cycles: 60%	[11]
Oxidized pulp fiber	Covalent binding	After 7 cycles: 60%	[17]
Porous hydroxyapatite/calcium alginate composite beads	Physical adsorption and Covalent binding	After 10 cycles: 40%	[21]
Fe_3O_4@SiO_2–NH_2	Covalent binding	After 7 cycles: 64.4%	[35]
Magnetic silica aerogel	Adsorption	After 10 cycles: 57%	Present work
Pure silica aerogel	Adsorption	After 10 cycles: 21%	Present work

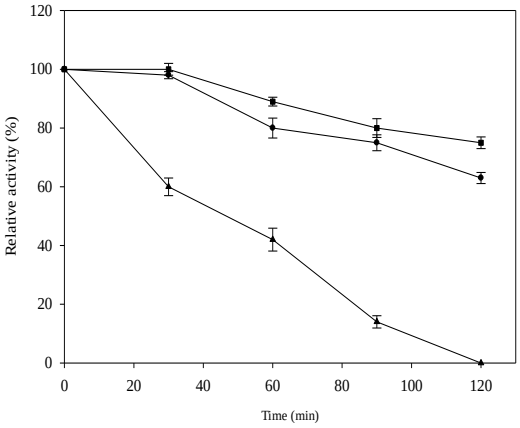

Fig. 10. Thermal stability of free and immobilized pectinase at 60℃. Free pectinase ▲, Pectinase adsorbed on pure silica aerogel ■ Magnetic silica aerogel●.

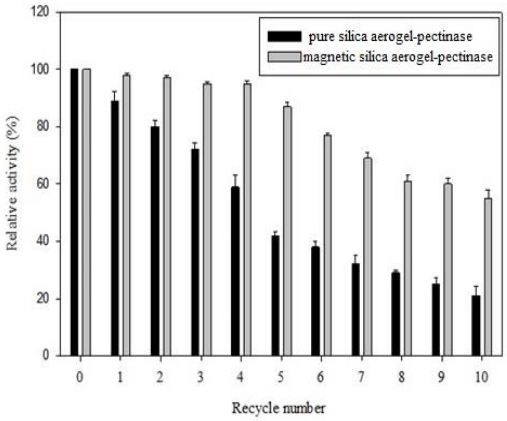

Fig. 11. Reusability of immobilized pectinase on pure silica aerogel ■ Magnetic silica aerogel■.

Reusability

The main advantage of the immobilization of an enzyme is that an expensive enzyme can be repeatedly used [34]. The reusability of theimmobilized enzyme was studied ten times and the results are shown in Fig. 11. It can be observed that the activity of the immobilized enzyme on pure silica aerogel was quickly decreased and after 5 times its activity reached half its initial activity and after 10 times its relative activities became 21%. As mentioned previously, pure silica aerogels are very porous and fragile. Thus they broke during handling and catalytic tests. This erosion led to the enzymes being desorbed and activity decreased. Desorption of enzymes could be observed after further repeated use.

In magnetic silica aerogels, theincorporation of the iron nanoparticles in aerogel pores improved the mechanical properties and prevented the cracking of composites. However, the operational stability of the immobilized enzyme was improved when theseparation of thebiocatalyst was done magnetically.

In Table 4, the reusability of immobilized pectinase of several related previous reports with the present study was compared. In these reports, the pectinase enzyme was immobilized in various supports using different immobilization methods. As shown in this table, the relative activity of pectinase-magnetic silica aerogel was higher than these reports.

CONCLUSIONS

In this study, pectinase was successfully immobilized on hydrophilic silica aerogel and its magnetic nanocomposite by physical adsorption. Characterization of

these supports before and after immobilization using BET, FESEM, FT-IR, and VSM analysis provided clear evidence of the adsorption of pectinase onto the support surfaces. The adsorption process was completed at a reaction time of 90 min for both supports. Immobilization yield for pure silica aerogel at all times was higher than magnetic silica aerogel. Also ,Kinetic studies and adsorption isotherms showed that the capacity and affinity of pure silica aerogel to pectinase molecules were higher than magnetic silica aerogel. However reusability experiments revealed that magnetic silica aerogel had better operational stability compared with pure silica aerogel. The thermal stability of both immobilized pectinases was improved considerably compared with free pectinase. The high maximum adsorption capacity and good affinity of silica aerogels showed that they are potential matrixes for pectinase immobilization applications because of their porous and hydrophilic nature. On the other hand, mechanical stability and ability to theseparation of these materials from the reaction medium could be improved with the incorporation of iron oxide particles in their pores.

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