Magnetic Biocatalysts of Pectinase: Synthesis by Macromolecular Cross-Linker for Application in Apple Juice Clarification

Running head: Magnetic biocatalysts for clarification of apple juice

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SUMMARY

Research background. Pectinase enzyme has become a valuable compound in beverage industry. One of the most significant concepts to overcome the drawbacks of using industrial enzymes is the immobilization of enzymes. In the present study, magnetic chitosan microparticles were utilized as a substrate for pectinase enzyme immobilization. New methods of enzyme immobilization involve the use of non-chemical cross-linkers between the enzyme and the substrate. The aim of this study was to immobilize the pectinase enzyme using polyaldehyde kefiran as a macromolecular crosslinker on magnetic particles.

Experimental approach. Pectinase enzyme was immobilized in four steps: relative oxidation of kefiran and its application as a crosslinker; production of magnetic iron (II, III) oxide (Fe₃O₄) microparticles; coating of magnetic Fe₃O₄ microparticles with chitosan; and immobilization of enzyme on the substrate, prepared by the use of oxidized kefiran crosslinker. Parameters, such as crosslinking concentration, time, and ratio of chitosan magnetic microparticles to enzyme, were optimized. Fourier-transform infrared spectroscopy (FTIR), dynamic light scattering (DLS), transmission electron microscopy (TEM), and vibrating sample magnetometer (VSM) were used to identify the groups and investigate the structures. The biochemical properties (stability of enzyme activity at different pH, temperature and time),
enzyme reusability, kinetics parameters (K_m and V_max) and apple juice turbidity, using free and immobilized pectinase enzymes, were also measured.

Results and conclusions. Cross-linker concentration, crosslinking time and ratio of CMMP to enzyme were important factors in activity recovery of pectinase enzyme. FTIR analysis correctly identified functional groups in the structures. The results showed that after enzyme stabilization, the particle size and molecular mass increased and decreased the magnetic saturation strength, respectively. According to the thermal kinetic study, the immobilized pectinase was higher than its free form. In the present study, findings indicate the excellent stability and durability of the immobilized pectinase. Findings indicate the excellent stability and durability of the immobilized pectinase in present study. Finally, a magnetic pectinase micro-biocatalyst was used to clarify apple juice, which reduced turbidity of processing.

Novelty and scientific contribution. This study investigates the usage of kefiran oxidized as a new cross linker for the immobilization of pectinase enzyme. Magnetic pectinase micro-biocatalyst has a good potential for industrial applications in the food industry, with high thermal stability.

Keywords: magnetic microparticles, pectinase, chitosan, kefiran, apple juice

INTRODUCTION

In recent years, juice production technologies have attracted major attention due to the increased consumption of natural juices in order to improve their quality (1). Freshly squeezed juice has a turbid appearance due to the colloidal dispersion of pectin (1.5 to 0.9 %) in the cell wall structure, which is one of the most important disadvantages of fruit juice processing (2). Pectic substances are a complex of glycoside macromolecules, with a high molecular mass. These substances can be found in plants and are responsible for the structure and integrity of plant tissue (3).

Several enzymes are used simultaneously to decompose pectic substances. Pectolytic enzymes or pectinases are a heterogeneous group of enzymes, involved in the decomposition of pectic substances (4). The main industrial applications of pectinases include extraction, clarification, and shredding of fruit and vegetable pulps (5). Commercial pectinases are usually a mixture of pectin esterase, polygalacturonase, and pectate lyase, each affecting part of the pectin chain and converting it to soluble compounds (6, 7). Pectinases as hydrolytic enzymes are used abundantly in different industries such as fruit juice extraction, coffee and tea fermentation, cotton scouring, water and wastewater treatment, and bleaching of paper (8).
The industrial enzymes and their applications are challenged due to restrictions associated with treatment factors or the enzyme, for instance substrate- and product-inhibitions, low operational stability, high production cost and difficult recovery (9). One of the most significant concepts to defeat these drawbacks is immobilization of the enzymes (10). Various benefits of immobilized enzymes include enhanced enantioselectivity and reusability of the enzymes, improved operational stability and enzyme environment, easier reactor operation, suitable product separation, and enzymes resistance to denaturation/ degradation/ aggregation (9, 11). The improved stability and reusability can significantly reduce the cost of an enzyme and thus make the industrial application economically feasible (12). If an improper immobilization is used, immobilized enzymes usually demonstrate lower activity than the free enzymes. This phenomenon leads to partially blocking of the enzymes’ active sites, the enhanced mass-transfer limitations between the enzymes and substrate, and conformational changes in the enzymes (9). In result, the selection of an appropriate immobilization strategy depends on the physical and chemical characteristics of the enzyme and immobilization matrix (10).

Enzyme immobilization methods include surface adsorption on organic polymers, metal oxides, and silica materials, entrapment in natural and synthetic polymers, ion exchange, crosslinking, and covalent bonding to organic polymers (11). Enzyme immobilization in the composite structure has always been challenging. Until now, various substrates have been used to immobilize enzymes (13). These substrates should have the following basic properties: availability, tendency to bind to proteins, presence of free working groups and reactions with the target enzyme, hydrophilic strength, mechanical stability, tissue stiffness, flexibility in different geometrical structures, permeability, and appropriate surface for transfer, degradability, cost-effectiveness, and safety of application (14, 15).

In the present study, magnetic chitosan microparticles were used as a substrate for pectinase enzyme immobilization. Cationic biopolymer chitosan, as an available and non-toxic compound, is the most renewable biopolymer after cellulose (16). This biopolymer and its derivatives have great potentials for application in different fields, such as agriculture, food, textile, cosmetic, and pharmaceutical industries, environmental protection, and biomedical research (17). It is known that chitosan solubility in aqueous solutions is limited due to the presence of strong hydrogen bonds and intermolecular reactions. However, its solubility in acidic environments (organic, mineral, and dilute acids) depends on crystallinity, polymerization, neutralization of amine groups, glucosamine distribution, solvent ion strength, pH, and concentration (16). Chitosan is formed by the protonation of amine groups of water-
soluble salts in repeating units (17).

Magnetic particles are particles of an independent nature with magnetic elements. Core/shell structured magnetic composite particles possess unique physicochemical characteristics and are dramatically different from their mass form (18).

The benefits of these particles include their ability for simple and rapid assembly, low cost, high loading capacity due to their large specific surface, presence of functional groups, and unlimited permeability in solutions (18). The utilize of magnetic particles for enzyme immobilization is a promising treatment strategy, as they can be easily recovered by external magnetic particle and recycled for further use (19). Previous studies have shown favorable results for the use of magnetic particles as a substrate for the immobilization of protease, catalase, phenylalanine ammonia lyase, lipase, beta-glucosidase, peroxidase, pullulanase, beta and alpha-galactosidase, laccase, glucoamylase and invertase enzymes (10, 18, 19, 20, 21).

One method of enzyme stabilization is to apply crosslinkers and create a strong link between the enzyme and the substrate (21). Different cross-linking agents are known and may be utilized. Although glutaraldehyde remains cheap and versatile, some enzymes are inactivated by this reagent, and toxic nature. In fact, its small size facilitates penetrating to the active site of the target enzyme catalytically causing inactivation and makes accumulation of the enzymes thereby covering their active sites then loss of activity (22). So, glutaraldehyde cannot be utilized widely in the food industry and using it is in contrast with the term green synthesis like polysaccharides (23). In previous studies, poly-aldehyde dextran, chitosan (M-CLEAs), and pullulan (PAP) were used as crosslinkers, respectively (17, 21, 23). Polysaccharides, produced in kefir grains, have a 1:1 glucose-to-galactose ratio, with a rotation angle of +68 (24). In the present study, oxidation-controlled kefiran was used as a non-chemical crosslinker. However, magnetic chitosan microparticles have not been investigated as substrates for pectinase immobilization by using polyaldehyde kefiran as a crosslinker; therefore, further investigation is needed in this area. It consists of hydroxyl groups that by partial oxidation provide the possibility of coupling between the enzyme and the carrier and using it as a safe, non-toxic and efficient crosslinker (25).

In the present study, pectinase enzyme was immobilized in four steps: (1) relative oxidation of kefiran and its application as a crosslinker; (2) production of magnetic iron (II,III) oxide (Fe₃O₄) microparticles; (3) coating of magnetic Fe₃O₄ microparticles with chitosan; and (4) immobilization of enzyme on the substrate, prepared by the use of oxidized kefiran crosslinker. Fig. 1 illustrated the steps of enzyme immobilization.
MATERIALS AND METHODS

Materials

Pectinase (lyophilized powder; EC 3.2.1.15) and chitosan (90.5 % degree of deacetylation) were purchased from Sigma-Aldrich (St-Quentin Fallavier, France). Kefir grains were obtained from a household in Tehran, Iran. In addition, iron (II) chloride tetrahydrate (FeCl$_2$·4H$_2$O, 99.7 %), iron (III) chloride hexahydrate (FeCl$_3$·6H$_2$O, 99 %), hydrochloric acid (HCl), sodium hydroxide (NaOH), sodium metaperiodate (NaIO$_4$), 2,4-dinitrophenylhydrazine (DNPH), apple pectin (100 Mw, 120 kDa, 85 to 90 % degree of esterification, 65 % galacturonic acid), and 3,5-dinitrosalicylic acid (DNSA) were acquired from Sigma-Aldrich Chemical Co. (Saint Louis, USA). All aqueous solutions were prepared with deionized water that had been passed through a Millipore Milli-Q Plus water purification system. Other chemicals were all analytical grade and used without purification. All other chemicals were of analytical grade.

Macromolecular crosslinking of polyaldehyde kefiran (PAK)

For partial oxidation of kefiran, sodium phosphate-buffered (SPB) NaIO$_4$ (c(NaIO$_4$): 100 to 600 mM) was added to polysaccharide kefiran as an oxidizing solution. Kefiran was kept and oxidized in a dark place for 30 to 150 min in an oxidizing solution. After oxidation, an ethylene glycol solution (0.3 mL) was added to terminate oxidation, and the PAK solution was kept at 4 °C overnight (26).

Determination of the oxidation rate of PAK

In this experiment, oxidized kefiran (0.3 %, 3×10$^{-4}$ g/L) was added to a freshly prepared solution of DNPH (10 mL, 1 % m/V). The mixture was kept at ambient temperature for 1 h and then centrifuged at 8000 × g for 10 min. The unreacted DNPH in the supernatant was measured using an ultraviolet spectrophotometer (Thermo Scientific, Madison, WI, USA) at 357 nm and calculated based on Equation 1 (27).

$$\text{Aldehyde content (mmol/g)} = \frac{n \text{Reacted DNP} \times (\text{mg/L})}{198.14} \frac{1}{m \text{amount of oxidized kefiran (g/L)}}.$$

Where 198.14 g/mol is the molecular mass of DNPH. The oxidized kefiran used as a macromolecular cross-linker. The oxidation rate of hydroxyl groups was calculated (28).
Synthesis of chitosan-Fe₃O₄ magnetic microparticles (CMMP)

First, 2 g of FeCl₃ was mixed with 1 g of FeCl₂ at a ratio of 2: 2 after dissolving in hydrochloric acid (0.5 mL). Next, the aqueous solution of FeCl₃ (45 mL) and FeCl₂ (45 mL) was added to a container with 80 mL of distilled water under nitrogen gas pressure. After stirring at 100 × g and adding 50 mL of 1.5 M NaOH, the solution reached pH of 10, and a black precipitate was produced. Next, iron oxide particles were removed from the medium using a magnet. In order to remove the additives, the particles were washed twice with distilled water and once with ethanol alcohol. At the end of this step, Fe₃O₄ magnetic microparticles (MMP) were produced. To cover 200 mL of microparticle suspension (0.25 %, m/V), 1 % chitosan solution and 45 mL of acetic acid 1 % were added, and the medium was completely sealed. Afterwards, Fe₃O₄ coating was carried out at 40 ± 3 °C for 2 h under constant stirring (200 × g) by overhead laboratory stirrer. The final magnetic product was separated by a permanent magnet and washed twice with distilled water. The final product was dried by freeze-drying and kept in dark conditions. The CMMP particles were dried overnight at room temperature and stored at low temperature for subsequent experiments (21, 29).

Immobilization of pectinase onto the carrier (Pectinase. PAK. CMMP: P. PAK. CMMP)

In the green synthesis, poly-aldehyde kefiran was utilized as a cross-linker rather than chemical cross-linkers comprising reactive aldehyde (-CHO) groups in the molecule. The aldehyde groups react with amine (-NH₂) groups to synthesis a Schiff base (N CH-) and this process was utilized by chitosan functional amine groups to cross-link with aldehyde kefiran groups (29). Magnetic chitosan microparticles were mixed (175 × g) with pectinase enzyme (10 mg protein) in SPB (100 mM, pH= 6) at a ratio of 1:1 to 1: 5 (m/m) and kept for 30 min. Polyaldehyde kefiran crosslinker (4 to 0.5 % of total solution) was prepared, and the mixture was incubated for 3 to 24 h. Next, sodium borohydride (5 mg) was added, and the mixture was kept for 30 min to terminate the process. The immobilized pectinase was magnetically separated, washed with buffer, and evaluated for enzyme activity. The P. PAK. CMMP was stored at 4 °C until the using time (31). The enzyme activity recovery is defined as the ratio of immobilized enzyme activity to total free enzyme activity of the start reaction (32): Equation 2:

\[
\text{Activity recovery (\%) } = \frac{\text{Immobilized enzyme activity}}{\text{Total enzymes activity}} \times 100\% \quad /2/
\]
Investigation of the structural and physicochemical properties of MMP, CMMP, and P. PAK.

CMMP

To confirm functionality, Fourier-transform infrared spectroscopy (FTIR) was applied using an FTIR spectrophotometer (Agilent Cary 660 series, Victoria, Australia), equipped with a deuterated-triglycine sulfate (DTGS) detector in the scanning range of 400 to 4000 cm$^{-1}$. FTIR provides an experimental and interpretive framework on the structure, interactions of natural polymers systems and physical characteristics. The surface charge and hydrodynamic size of the particles were measured using dynamic light scattering (DLS) method (Malvern Instruments Ltd., Worcestershire, UK). The volume size distribution was calculated from the intensity of light diffracted at each angle using theory. Transmission electron microscopy (TEM; JEOL 2100, Leoben, Austria) was also used to acquire images for demonstrating the surface morphology and structure of the particle solution. The effect of magnetic susceptibility and coating on the paramagnetic properties of synthesized particles was determined using vibrating sample magnetometer at room temperature (VSM, Kashan, Iran).

Evaluation of pectinase enzyme efficacy

Pectinase enzyme (0.5 mL) was incubated with 1 % apple pectin solution (prepared in phosphate buffer; pH= 6, 100 mM) for 20 min at 50 ± 2 °C. Next, DNSA reagent (2 mL) was added to the mixture, boiled for 15 min, and then cooled. The absorbance intensity of the mixture was measured at 575 nm with an ultraviolet spectrophotometer (Thermo Scientific, Madison, WI, USA). The enzyme activity was determined by the amount of enzyme required to release one mole of beta-galacturonic acid per min under optimal conditions (50 ± 2 °C, pH= 6). The protein concentration of the supernatant was measured using Bradford reagent with standard bovine serum albumin (1 mg/mL).

Investigation of biochemical properties of free and immobilized pectinase enzymes

Biochemical properties of pectinase enzymes were determined by investigation of the stability of enzyme activity in different acidic, alkaline, and temperature conditions by incubating a certain amount of free and immobilized enzymes under variable pH conditions (range: 2 to 8) at constant temperature (20 to 80 °C) or different temperatures with constant pH. Also, enzyme activity was measured within five-day intervals under standard conditions at constant pH (pH= 4) and temperature (20 °C) for a shelf-life of 30 days.
Reusability of immobilized pectinase enzyme

Reusability of immobilized pectinase was measured by reducing its activity after each application in the reaction medium for hydrolysis of the substrate. At the end of each step, the enzyme was separated from the reaction medium using a magnet. At the end of each step, the enzyme was separated from the reaction medium using a magnet and washed with phosphate buffer (100 mM, pH 6.0). Then, this enzyme was re-suspended in substrate solution to measure its activity and the process was repeated 10 cycles (N). With regard to residual activity, the activity was measured after each cycle (100 % residual activity for the first cycle) (33).

Kinetics of the free and immobilized pectinase activity

The kinetic parameters for both free and immobilized pectinases were evaluated using the Michaelis-Menten (MM) and Lineweaver-Burk (LB) plots. This was carried out by plotting different concentrations of pectin solution (2.0 to 8.0 mg/mL) against reaction velocity (Equation 3):

\[
\text{Velocity}(v) = \frac{V_{\text{max}} S}{K_m + S}
\]

Where, S is the substrate concentration (mg/mL), Vmax is the maximum reaction rate attained at an infinite substrate concentration (μmol of galacturonic acid/min), and Km is the constant rate (mg/mL) (6).

Application of immobilized pectinase in apple juice clarification

The potential of P. PAK. CMMP for clarification of apple (Var. Golab) juice was examined. The Fresh apple juice was centrifuged at 5000 × g for 20 min, and the supernatant was used for the clarification process. The immobilized pectinase (with an equivalent amount to free pectinase) was mixed with diluted juice (20: 80 v/v of juice in distilled water) and treated for 150 min at 50 °C. After enzymatic treatment, juice clarity was determined in terms of turbidity, using a spectrophotometric method (26).

Statistical analysis

Data presented in various studies were plotted using OriginPro software (Version 9. 4, Origin Lab. Corporation) and expressed as standard error (±). Each value represents the mean of three independent experiments, with an average standard deviation of < 5 % (34).
RESULTS AND DISCUSSION

Results of controlled oxidation of crosslinked polyaldehyde kefiran

The kefiran crosslinking macromolecule was prepared by controlled oxidation of metaperiodate. Periodic acid and its salts can specifically oxidize kefiran. The rate of oxidation to produce the maximum amount of aldehyde group is dependent on the concentration of NaIO₄ and reaction time. Therefore, the effects of NaIO₄ concentration and reaction time on the produced aldehyde groups were investigated based on the dinitrophenylhydrazine test (Fig. 2a).

An increase in the concentration of NaIO₄ increased the amount of aldehyde group to a maximum concentration of 500 mM within 120 min. However, at concentrations above 500 mM, the amount of polyaldehyde kefir decreased. Kefir oxidation may be inhibited at higher concentrations of NaIO₄. Also, longer reaction time did not significantly increase oxidation; in other words, the amount of OH groups accessible for aldehyde was reduced to 150 min. Finally, the oxidized kefiran (12.5 mmol/g) was dissolved in SPB (100 mM, pH= 6) and used as the kefiran cross-linking macromolecule. According to the method described by authors, the oxidation rate of hydroxyl groups was 35.71 % in the present study (21, 28).

Immobilization of pectinase onto P. PAK. CMMP

Synthetic cross-linkers such as glutaraldehyde and carbodiimide, have been used in the past. Nowadays, they are less used because of their small size and toxicity. They are also considered harmful to living organisms because of their toxic properties; therefore, they cannot be applied in the food industry. In recent years, many authors have used dextran, xanthan, pectin, and dextran aldehyde as natural crosslinkers instead of synthetic crosslinkers and reported positive results (23).

The aim of present study was to immobilize pectinase enzyme by using polyaldehyde kefiran as a macromolecular crosslinker on magnetic particles. Reactive aldehyde groups of oxidized kefiran can form amino groups in lysine or hydroxyl lysine of the enzyme, as well as chitosan group in the Schiff base, which in turn prevents enzyme removal during the reaction. The crosslinking parameters include the concentration and time of crosslinking, which have direct effects on enzyme loading, activity recovery, and operational stability of the prepared magnetic biocatalyst. Overall, to optimize the rate of crosslinking between the enzyme and magnetic particles, maximum recovery of enzyme activity is required (35, 36).
The effect of crosslinker concentration (\( \varphi \)) on the recovery of pectinase enzyme activity in the immobilized form was investigated by varying the concentration in the range of 0.5 % to 4 % (Fig. 2b). It was indicated that the amounts of cross-linkers had remarkable effects on the activity recovery process (37). The results showed that at a low crosslinker concentration (0.5 %), activity recovery decreased due to reduced enzyme crosslinking. In fact, low concentrations do not have the required capacity for the Schiff bond (covalent bond), and pectinase activity improved by increasing the concentration. In the present study, maximum activity recovery was observed for MMP, using 1.5 % polyaldehyde kefiran. In addition, an increase in the crosslinker concentration resulted in enzyme reorganization, denaturation, and subsequent activity loss. The previous reports showed that in excess of glutaraldehyde might produce the formation of clusters with mass transfer limitations, which resulted in lower activity recovery (38, 39).

Crosslinking time is another important factor in enzyme immobilization (32). In this study, as the crosslinking time advanced, the recovery of enzyme activity increased (Fig. 2c). Maximum recovery of pectinase immobilization activity in MMP has been observed within 12 h of linking to aldehyde kefiran. Further increase in the crosslinking time resulted in a gradual decrease in pectinase activity recovery due to excessive crosslinking, which led to the chemical modification of enzyme (30). Also prolonged crosslinking time could restrict the enzyme flexibility and abolish enzyme activity due to more intensive crosslinking (39).

To maximize pectinase immobilization, the ratio of chitosan magnetic particles to enzyme (1: 1 to 5: 1) was optimized (Fig. 2d). At the lowest ratio, the amount of CMMP was insufficient for pectinase loading, leading to its decreased activity. As the ratio increased, the activity recovery also enhanced significantly, depending on the type of microparticle. In CMMP, the particle-to-enzyme ratio of 3: 1 showed the highest activity recovery in pectinase enzyme. However, as the CMMP-to-enzyme ratio increased further, the activity recovery remained constant, possibly due to maximum enzyme loading onto the carrier (4). In the present study, increasing the values of these three factors on the rate of reactive recovery is in line with the results of some researchers (26, 37, 38, 39).

**Formation of MMP, CMMP, and P. PAK. CMMP**

The structure of MMP (a), CMMP (b) and P. PAK. CMMP (c) was determined using FTIR in the \( \tilde{\nu} \) range of 4500 to 500 cm\(^{-1} \) (Fig. 3).

The specific peak at 576.03 cm\(^{-1} \) was related to the intrinsic tensile vibrations of Fe–O in
the Fe$_3$O$_4$ structure (Fig. 3a). This peak was observed in all structures (a, b and c).

When chitosan was used as a coating for microparticles, peaks similar to native chitosan were observed. The peaks nearly 3385.07 cm$^{-1}$ were related to tensile vibrations O–H and NH (-NH$_2$), and peaks nearly 1068.56 cm$^{-1}$ were indicated N-H bend of R- NH$_2$ and C–O–C. In addition, the peaks at 2883.58 and 1643.35 cm$^{-1}$ were attributed to the tensile vibrations of C–H and N–H, respectively (Fig. 3b), which confirmed the successful preparation of CMMP (40, 41). These peaks were observed in a and b structures.

The structure of the final complex containing magnetic microparticles, chitosan, oxidized kefir and pectinase enzyme is shown (Fig. 3c). After enzyme immobilization, the peaks of diagrams a and b are still present, but some peaks are slightly altered or overlapped due to enzyme loading and the presence of new linker groups. The peaks at 869.01, 892.11, 927.37, and 984.40 cm$^{-1}$ indicated the presence of glucose, galactose, and beta crosslinker in the structure of polyaldehyde kefiran polymer, respectively (25). Also, the peak at 576.02 cm$^{-1}$ was related to the Fe$_3$O$_4$ structure. In addition to the peaks associated with the structure of kefiran polymer, chitosan and iron oxide peaks in the range of 1550.22 to 1650.05 cm$^{-1}$ indicated the presence of amino acids in pectinase enzyme (P. PAK. CMMP).

The magnetic saturation (σ) for MMP (a), CMMP (b), and P. PAK. CMMP(c) was 32.70, 19.90, and 20.15 A m$^2$/kg, respectively (Fig. 4). The two diagrams were roughly uniform, but could be deduced from the hysteresis rings. Overall, high magnetic saturation indicated high magnetizability, which suggests adequate isolation with conventional magnets in the present study. The results showed that after enzyme stabilization, the particle size and molecular mass increased and decreased the magnetic saturation strength, respectively. In other words, the higher the particle mass is, the lower the magnetic saturation strength will be; this finding is in line with previous research (42).

Since residual magnetization and forcing magnets were close to zero for almost all three samples, they exhibited behaviors similar to superparamagnetic particles and showed adequate capacity to disperse after the magnetic field is removed. Magnetic particles can easily separate the immobilized enzyme by using the reaction magnetic field due to its high magnetization (43).
The results of particle size analysis (d) showed that the particle size of MMP was about 1 μm in the first step, which increased to almost 4 μm in the final step (Fig. 5a); this is attributed to the large particle size from the beginning of the process (Fig. 5b), which increased by adding each coating around the primary microparticles (Fig. 5c).

The TEM results of MMP samples (Fig. 6a) showed that their spherical structure is almost regular and coherent, with a smooth surface. In CMMP samples (Fig. 6b), chitosan was well-seated on the particles. Increasing the size of PPAK CMMP particles (2805 to 3904 nm) relative to the previous particles indicates the linking of enzyme to particle, as demonstrated in Fig. 6c. Also, rough surfaces were observed considering the magnetic properties and size of particles.

Biochemical properties of the immobilized enzyme (temperature, time, and pH stability)

The relative stability of free and immobilized pectinase enzymes on CMMP in different pH ranges (2 to 8) is shown in Fig. 7a. The optimal pH was four for free and immobilized enzymes on MMP. At the highest pH (i.e., 8), free and immobilized pectinase activities on the microparticles were 20.06 % and 25.51 %, respectively. The immobilized enzyme on MMP had a significantly higher relative activity, compared to the free enzyme. Overall, magnetic particle substrates facilitate pectinase activity on pectin. Also, the possibility of enzyme form disruption and non-linking of enzyme to the pectin substrate reduces at suboptimal pH conditions; these findings are consistent with the results reported by researchers (6).

Stability of the relative activity of free and immobilized pectinase enzymes on CMMP at different temperatures is shown in Fig. 7b. The optimal temperature for free and immobilized enzymes on MMP was 50 °C and 60 °C, respectively. At the highest temperature (80 °C), activities of free and immobilized pectinase enzymes on the microparticles were 32.10 % and 40.10 %, respectively. According to the present results, at high temperatures (e.g., 80 °C), free and immobilized enzymes showed insignificant differences. On the other hand, significant differences were observed between the samples at 30 to 70 °C, and immobilized enzymes exhibited higher thermal resistance. Moreover, the optimal temperature for immobilized enzyme activity increased by 10 °C, that is, it shifted from 50 to 60 °C, and the enzyme showed resistance to denaturation. However, at temperatures above 60 °C, the links between the
crosslinker, enzyme, and magnetic particles were probably separated, and the relative activity of the target enzyme reduced (6, 44).

Another reason for the increased thermal stability of the immobilized enzyme is water depletion in the immobilized phase, which plays an important role in hydrophobic reactions, polarity reduction, and hydrophobic accumulation of proteins in the aqueous solution (45). The rate of enzyme loading and linkage in the microparticles was not very high. Therefore, the heat resistance was investigated because the particles are more closely linked to each other at high temperatures, filling the active sites of enzyme and reduce its activity. The rate of heat resistance of β-galactosidase enzyme, immobilized on the microparticles was determined (46).

Fig. 7

Furthermore, the storage stability of free and immobilized pectinase enzymes was determined within 5-day intervals for 30 days. The results showed that after one month, the residual activity of free pectinase enzyme was 32.11 %, while 56.47 % of residual activity was related to the microparticles in the immobilized state (Fig. 7c). Appropriate stability of immobilized pectinase storage may be due to crosslinking by polyaldehyde kefiran on chitosan magnetic particles, which prevents the effects of possible accumulation at the active sites of enzyme (43).

Reusability of enzyme

The diagram of the relative activity of the immobilized pectinase enzyme on CMMMP with repeated applications is presented in Fig. 7d. Generally, enzymes are widely used in the food industry because of their activity as catalysts; however, they are expensive considering their reuse. Therefore, new methods of enzyme immobilization have been suggested for the reuse of enzymes. In the present study, removal of the immobilized pectinase enzyme by magnetic chitosan particles was investigated after catalytic activity and reuse of the enzyme 10 times.

Determination of the number of reusable loads per enzyme is a key factor in cost-effective application of immobilized enzymes (34). However, reduced enzyme activity after repeated use is related to enzyme denaturation or loss of enzymatic links and crosslinkers (6). The decrease in the relative activity of enzyme after reuse is probably due to the release and separation of pectinase enzyme from the substrate by magnetic chitosan, which occurred due to separation and washing in each step; however, reduction of relative activity in CMMMP after 10 repeated applications reached 57.11 %. The results showed that magnetic particles were
more successful in maintaining this property.

**Kinetic parameters of free and immobilized enzymes**

The kinetic parameters demonstrated that the $K_m$ of the free enzyme (3.201 mg/mL) was higher than the immobilized enzyme (2.713 mg/mL). The substrate remained after the immobilized enzyme by cross-linker. The enzyme tendency was high to connect to substrate because the flexibility remainder even after cross-linking (6, 23, 47).

A decrease in $V_{max}$ for free pectinase (0.595 μmol of galacturonic acid/min) compared with the $V_{max}$ for immobilized pectinase (0.924 μmol of galacturonic acid/min) showed that the rate of pectin hydrolysis increased after immobilization (6). It is along the linear chain due to the covalent cross-linking between poly-aldehyde kefiran and pectinase enzyme. So, it inhibits the constitute of enzymatic molecular mass due to the unchanged mass transfer from the matrix surface (23, 47).

**Clarification of apple juice by P. PAK. CMMP**

The turbid appearance of fruit juice is attributed to the presence of pectic components. Accordingly, breakdown of these polysaccharides by pectinase can improve the quality and storage stability of apple juice (48). In the current study, apple (Var. Golab) juice was treated using immobilized and free pectinase enzymes. Juice clarification was measured in terms of turbidity reduction, using the spectroscopic method (Table 1).

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At the beginning of clarification process, the rate of turbidity reduction by free pectinase enzyme was found to be 100 to 87 %, whereas the immobilized pectinase enzyme exhibited 100 to 90 % turbidity reductions within 30 min. As time progressed, free pectinase treatment reduced the turbidity of apple juice up to 70 %, while immobilized pectinase treatment reduced turbidity to 75 % after 120 min of treatment.

**CONCLUSIONS**

The results of the present study showed the potential use of polyaldehyde kefiran as a crosslinking agent for immobilization of pectinase on CMMP. Our findings showed that the most appropriate rate of polyaldehyde kefiran crosslinker was 1.5 % within 12 h of contact time; the highest recovery rate of pectinase activity was observed at a CMMP-to-enzyme ratio.
of 3: 1. Particle size changes ranged from 1000 (MMP) to 4000 nm (P. PAK. CMMP). The results of pectinase immobilization showed improvements in the biochemical properties (i.e., temperature, time, and pH stability) and apple juice clarification parameters. It can be concluded that magnetic pectinase micro-biocatalyst, with high thermal stability, has a good potential for industrial applications in food industry.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS’ CONTRIBUTION

M. Nouri: data collection, data analysis and interpretation, drafting the article
F. Khodaiyan: design of the work, performing the analysis, critical revision

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Table 1. Clarification of apple juices by free and immobilized pectinase.

<table>
<thead>
<tr>
<th>t/min</th>
<th>Turbidity/%</th>
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<tbody>
<tr>
<td></td>
<td>Free pectinase</td>
</tr>
<tr>
<td>0</td>
<td>100± 1.10</td>
</tr>
<tr>
<td>30</td>
<td>87± 2.57</td>
</tr>
<tr>
<td>60</td>
<td>81± 3.01</td>
</tr>
<tr>
<td>90</td>
<td>76± 2.10</td>
</tr>
<tr>
<td>120</td>
<td>70± 3.09</td>
</tr>
</tbody>
</table>
Fig. 1. Scheme of enzyme immobilization steps
Fig. 2. (a) Aldehyde content generated in kefiran after oxidation with meta periodate (100–600 mM) determined by DNPH method. Effect of cross-linker concentration (b), cross-linking time (c) and ratio of CMMP to enzyme (protein) (m/m) (d) on activity recovery of pectinase in magnetic microbiocatalyst. The measurements were performed in triplicate and the error bar represents the percentage error.
Fig. 3. FTIR spectra of (a) the MMP, (b) CMMP, and (c) P.PAK.CMMP.

Fig. 4. Magnetic hysteresis loops of the (a) MMP, (b) CMMP, and (c) P. PAK. CMMP.
Fig. 5. DLS curves of the (a) MMP, (b) CMMP, and (c) P. PAK. CMMP

Fig. 6. TEM images of the (a) MMP, (b) CMMP, and (c) P. PAK. CMMP
Fig. 7. (a) Investigation of different pH 2 - 8 (temperature constant= 40 °C) (b), and temperatures of 20-80 °C (pH constant 4) (c) stability of different time 0-30 d of pectinase immobilized onto PAK. CMMP and free enzyme, (d) reusability of P. PAK. CMMP 1 during 10 cycles