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Research Article

Preparation and characterization of κ-carrageenase immobilized onto magnetic iron oxide nanoparticles

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ABSTRACT

Background: Carboxyl-functionalized magnetic nanoparticles were synthesized via chemical co-precipitation method and modified with oleic acid which was oxidized by potassium permanganate, and κ-carrageenase from *Pseudoalteromonas* sp. ASY5 was subsequently immobilized onto them. The immobilization conditions were further optimized, and the characterizations of the immobilized κ-carrageenase were investigated.

Results: The κ-carrageenase was immobilized onto magnetic iron oxide nanoparticles, and the bonding was verified by Fourier transform infrared spectroscopy. The optimal conditions for κ-carrageenase immobilization were 2.5% (w/v) glutaraldehyde, 13.9 U κ-carrageenase for 20 mg of magnetic nanoparticles, a 2-h cross-linking time, and a 2-h immobilization time at 25°C. Under these conditions, the activity of the immobilized enzyme and the enzyme recovery rate were 326.0 U · g^{-1} carriers and 46.9%, respectively. The properties of the immobilized κ-carrageenase were compared with those of the free enzyme. The optimum temperatures of the free and immobilized κ-carrageenase were 60 and 55°C, respectively, and the optimum pH of κ-carrageenase did not change before and after immobilization (pH 7.5). After immobilization, κ-carrageenase exhibited lower thermal stability and improved pH stability, as well as better storage stability. The immobilized κ-carrageenase maintained 43.5% of the original activity after being used 4 times. The kinetic constant value (Km) of κ-carrageenase indicates that the immobilized enzyme had a lower binding affinity for the substrate.

Conclusions: Under optimal conditions, the activity of the immobilized enzyme and enzyme recovery rate were 326.0 U · g^{-1}·κ-carrageenase-CMNPs and 46.9%, respectively. The thermal, pH, and storage stabilities of κ-carrageenase-CMNPs were relatively higher than those of free κ-carrageenase.

1. Introduction

Carrageenans are gel-forming, linear, and sulfated galactans extracted from certain marine red algae and consist of D-galactose residues with alternating α-1,3 and β-1,4 linkages [1]. Recent studies have shown that carrageenan oligosaccharides with small molecular weight possess certain biological activities, such as anti-oxidation [2], anti-viral [3], anti-angiogenic [4], anti-inflammatory [5], and immunomodulation [6], compared with other carrageenans. Therefore, carrageenans are often degraded into oligomers by using acid, active oxygen, microwave, sonication, or carrageenase to improve their bioactivity and application performance [7].

Compared with chemical and physical degradation, enzyme hydrolysis is more likely to produce carrageenan-derivatives with uniform molecular weights, which can be more advantageous because the observed physiological activities of oligo-carrageenans are associated with their molecular weights [8]. However, the industrial application of the enzyme as a biocatalyst is economically unattractive because of its high cost and inconvenience in separation, recycling, and reusing [9]. Enzyme immobilization presents interesting advantages over these problems, including highly concentrated enzymatic activity and high stability and reusability. Tang and Lee [10] immobilized β-glucosidase on κ-carrageenan hybrid matrix, and the immobilized enzyme tolerated broader range of pH values and higher reaction temperature up to 60°C compared with the free β-glucosidase. Bezerra et al. [11] immobilized laccase in green coconut fiber, and the immobilized enzyme retains up to 100% of the initial activity after the assay was reused 10 times. Among the various supports used in immobilization, the magnetic nanoparticles are

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receiving considerable attention because of their large specific surface area, low mass transfer resistance, less fouling, easy separation from reaction solution with magnetic field, less capital and operating costs, and surface modification with various active groups [12,13,14].

To our knowledge, κ-carrageenase immobilization with magnetic nanoparticles has not been reported yet. In the present study, carboxyl-functionalized magnetic nanoparticles were synthesized via chemical co-precipitation method, and κ-carrageenase was subsequently immobilized onto carboxyl-functioned magnetic iron oxide nanoparticles (CMNPs). The immobilization conditions were further optimized, and the characterizations of the immobilized κ-carrageenase were investigated.

2. Materials and methods

2.1. Materials

$FeCl_3 \cdot 6H_2O$, $FeCl_2 \cdot 4H_2O$, NH_4OH, $KMnO_4$, oleic acid, 3,5-dinitrosalicylic acid, and potassium sodium tartrate were of analytical grade and purchased from Sinopharm Chemical Reagent Ltd., Corp. (China). κ-Carrageenan was provided by Greenfresh (Fujian) Foodstuff Co. Ltd.

2.2. Microorganism and κ-carrageenase preparation

Pseudoalteromonas carrageenovora CICC 23819, deposited at the China Center of Industrial Culture Collection, was used to produce κ-carrageenase. κ-Carrageenase production was performed by shaking flask fermentation using carrageenan as the sole carbon source. The flasks were incubated at 20°C for 60 h in a shaker at 180 rpm. Culture supernatant as crude enzyme was collected by centrifugation at 9800 × g and 4°C for 10 min.

2.3. Synthesis of oleic acid-coated magnetic nanoparticles and CMNPs

Oleic acid-coated magnetic nanoparticles were prepared via chemical co-precipitation method as described by Chen et al. [13]. $FeCl_3 \cdot 6H_2O$ (8.1 g) was dissolved in a flask with 142.5 mL of distilled water and then heated to 70°C. $FeCl_2 \cdot 4H_2O$ (3.3 g) was dissolved in 7.5 mL of water, and the mixture was subsequently added into the flask. Under rapid stirring condition, 18 mL of ammonia (25%, w/v) was quickly added. After 1 min, 5.3 mL of oleic acid was added dropwise into the flask and maintained at 70°C for 1 h under continuous stirring. The black precipitate was separated using an external magnet and washed several times with alcohol and water to remove the excess oleic acid.

Thereafter, 160 mL of 10 g · L^{-1} $KMnO_4$ solution was added into the oleic acid-coated magnetic nanoparticles obtained earlier. The precipitate was separated using a magnet after 8 h of sonication treatment and washed several times with water to remove excess KMnO4. Powdered CMNPs were obtained after drying at ¯80°C under vacuum.

2.4. Immobilization of κ-carrageenase onto CMNPs

CMNPs (20 mg) were added into 5 mL glutaraldehyde solution (5%, w/v) and incubated at 4°C for 3 h for cross-linking. The magnetic nanoparticles were separated magnetically and washed five times with sodium phosphate buffer (50 mM, pH 7.5) to remove the excess glutaraldehyde. Subsequently, the mixture of the supports obtained above and 1 mL of κ-carrageenase solution were immobilized at 4°C for 3 h. CMNPs bound with κ-carrageenase were collected using an external magnetic field, washed with sodium phosphate buffer (50 mM, pH 7.5), dried at ¯80°C under vacuum using a freeze dryer, and stored at 4°C for activity assay.

2.5. Enzyme activity assay

The activities of free κ-carrageenase and κ-carrageenase-CMNPs were determined using the 3,5-dinitrosalicylic acid (DNS) method. The substrate used was 0.5% (w/v) κ-carrageenan dissolved in sodium phosphate buffer (50 mM, pH 7.5); the resulting solution was uniformly mixed with carrageenase and incubated at 55°C for 20 min. Subsequently, the DNS was added into the reaction solutions and incubated in boiling water for 10 min; the absorbance was measured at 520 nm thereafter. The amount of sugar was determined based on the D-galactose in standard curve. One unit of κ-carrageenase activity is defined as the amount of enzyme that produced 1 μmoL of D-galactose in 1 min at 55°C and pH 7.5.

Enzyme recovery rate was calculated according to the residual enzyme activity after immobilization. Free κ-carrageenase and κ-carrageenase-CMNP activities were assayed, and the enzyme activity recovery rate was calculated based on the following equation:

$$\text{Activity recovery rate } (\%) = \text{ai}/\text{af} \times 100\% \quad \text{[Equation 1]}$$

where ai is the activity of the immobilized κ-carrageenase, and af is the activity of the free κ-carrageenase added into immobilization system.

2.6. Determination of optimal conditions for κ-carrageenase immobilization

Glutaraldehyde concentration (0%–3.5%, w/v), enzyme dosage (6.95–83.4 U κ-carrageenase mL^{-1}), cross-linking time (0.5–6 h), immobilization time (0.5–6 h), and immobilization temperature (4 and 25°C) were investigated to determine the optimal immobilization conditions of κ-carrageenase. The immobilization efficiency during the immobilization process was evaluated using the activity recovery rate of κ-carrageenase.

2.7. Fourier transform infrared spectra (FTIR) of CMNPs and κ-carrageenase-CMNPs

FTIR spectra were obtained on a NEXUS 670 FTIR instrument using KBr discs in the 4000–400 cm^{-1} region at room temperature (25°C).

2.8. Properties of the free and immobilized κ-carrageenase

The effect of temperature on the κ-carrageenase activity was determined at various temperature levels from 45°C to 65°C, pH 7.5. The effect of pH on κ-carrageenase activity was assayed at 55°C with pH values that ranged from 4.0 to 10.0 in the following buffers: acetic acid-sodium acetate (50 mM, pH 4.0–5.0), Na_2HPO_4–NaH_2PO_4 (50 mM, pH 5.0–7.0), Tris–HCl (50 mM, pH 7.0–9.0), and glycine–sodium hydroxide (50 mM, pH 9.0–10.0). The maximum activity was considered to be 100% and used as the reference to determine the relative activity at different catalytic reaction conditions.

The thermal stability of κ-carrageenase was evaluated by measuring the residual activity of the free and immobilized enzyme after incubation from 45°C to 60°C without substrate. The residual activities of the free and immobilized enzymes were assayed after incubating κ-carrageenase at 4°C for 24 h in the aforementioned pH buffers to determine pH stability. The residual enzyme activity was defined as the proportion of the final value of enzyme activity to the initial activity (100%).

2.9. Reusability and storage stability assays

The reusability of the immobilized κ-carrageenase was evaluated by repeated utilization to catalyze the κ-carrageenan hydrolysis at 55°C and pH 7.5. The activity obtained in each round was compared with the initial activity (defined as 100%) to calculate the relative activity.

The storage stability was assessed by storing the free and immobilized κ-carrageenase at 4°C for 30 d.

2.10. Kinetic parameters

Kinetic parameters (Km) of free and immobilized κ-carrageenase at optimum temperature were determined using different κ-carrageenan concentrations from 0.3% to 1.0% (w/v) κ-carrageenan at 55°C with pH 7.5.

3. Results and discussions

3.1. Immobilization of κ-carrageenase

The magnetic nanoparticles obtained using the co-precipitation method exhibited a strong magnetic response and superparamagnetic property. The prepared CMNPs were employed to immobilize κ-carrageenase using glutaraldehyde as the cross-linking agent. The immobilization efficiency and immobilization enzyme were 46.9% and 326.0 U · g^{-1} magnetic nanoparticles, respectively.

3.2. FTIR spectroscopy

FTIR determined the structures of CMNPs, κ-carrageenase-CMNPs, and free κ-carrageenase (Fig. 1). The vibrational peaks at 571 cm^{-1} on the CMNPs and κ-carrageenase-CMNPs were attributed to the Fe–O bond, and it was the characteristic absorption band for Fe3O4. Peaks around 2860 and 2925 cm^{-1} on the CMNPs and κ-carrageenase-CMNPs were due to the stretching of –CH2 and C–H in the oleic acid. A band at 1640 cm^{-1} was attributed to the bending vibrations of the –OH groups in water molecules or carboxyl groups [15]. Fig. 1 also shows a large band around 3420 cm^{-1}, probably due to the physical adsorption of water or the presence of NH_2 groups [16]. Peak around 1100 cm^{-1} on the κ-carrageenase-CMNPs and κ-carrageenase were due to the stretching of C–N from peptide bond in protein. However, this peak is not present in CMNPs. The results showed κ-carrageenase were successfully immobilized on CMNPs.

3.3. Effects of immobilization parameters

Glutaraldehyde as a bifunctional cross-linking agent is generally used in inter- and intra-molecular cross-linking reactions [17]. However, excessive cross-linking may lead to aggregation, precipitation, loss of activity, and distortion of the 3D enzyme structure [18]. Therefore, glutaraldehyde concentration is an important factor for enzyme immobilization. The effects of glutaraldehyde concentration on the immobilization activity and efficiency were investigated at 0%–3.5% (Fig. 2). As a result of the bonding between the aldehyde and amine groups of κ-carrageenase, the highest enzyme activity of 278.6 U · g^{-1} carries was obtained when glutaraldehyde concentration reached 2.5%. A slight decrease in the activity of the immobilized κ-carrageenase was observed above 2.5% glutaraldehyde. This finding is probably due to the excessive glutaraldehyde that may cause unavoidable chemical modification and denaturation effects [19]. Another possible reason for this finding is that the active sites of the enzyme for substrate may be blocked by the carrier and other proteins [20].

The enzyme concentration is an important factor that affects the enzyme recovery rate during the immobilization process. Enzyme solutions (6.95 U · mL^{-1} to 83.4 U · mL^{-1}) were used to analyze the effect of enzyme concentration on κ-carrageenase immobilization. Fig. 2 shows that the immobilized κ-carrageenase activity gradually increased with increasing initial enzyme concentration. When the magnetic nanoparticles reached its saturation adsorption capacity, the activity of the immobilized κ-carrageenase reached the highest value (326.9 U · g^{-1} carries) and still remained stable. In contrast to the trend of the enzyme activity, the enzyme recovery rate rapidly declined with the increasing concentration of κ-carrageenase. The κ-carrageenase concentration of 13.9 U · g^{-1} was selected for 20 mg of magnetic nanoparticles to ensure the enzyme activity and recovery rate.

The influence of cross-linking time on the activity of the immobilized enzyme is shown in Fig. 2. The highest enzyme activity recovery rate observed was 44.8% (311.2 U · g^{-1} carries) for 2 h of cross-linking. Less cross-linking time may lead to rest of binding sites on the CNMPs, which can result in low enzyme activity. By contrast, prolonged cross-linking time may lead to a steric hindrance among the enzyme molecules, thereby blocking the active sites over CMNPs and making the binding sites for the substrate unavailable toward the enzyme [21]. Therefore, the cross-linking time was set for 2 h to obtain maximum activity recovery rate.

Immobilization time was also an important factor to determine the amount of enzyme immobilized on the magnetic nanoparticles and may finally affect the activity of the immobilized κ-carrageenase. Fig. 2 shows that the enzyme activity gradually increased to 359.5 U · g^{-1} carries during the first 2 h. However, the enzyme activity decreased to only 318.9 U · g^{-1} carries after 6 h of immobilization because of the denaturation effect. The recovery rate showed a similar tendency to the enzyme activity, reaching its maximum at 2 h.

Temperature is a significant factor that affects the catalytic activity and stability of the enzyme. The effect of temperature on enzyme immobilization was investigated (Fig. 2). The immobilization process at 25°C could obtain activity as high as that at 4°C.

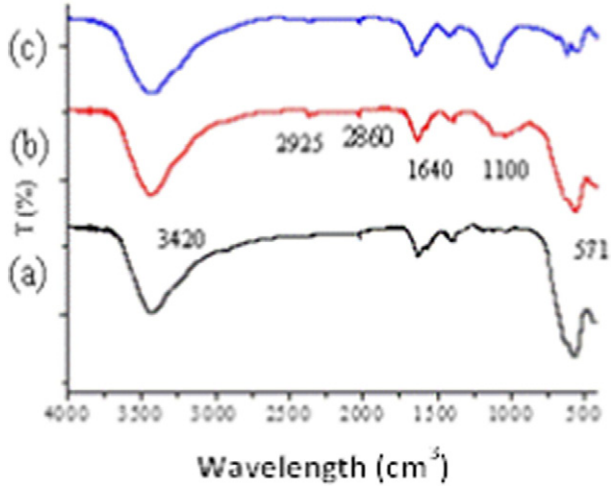

Fig. 1. FTIR spectra of CMNPs (a), κ-carrageenase-CMNPs (b), and free κ-carrageenase (c).

3.4. Effects of temperature and pH on the activities of free and immobilized κ-carrageenase

The optimum temperatures of the activities of free and immobilized κ-carrageenase were investigated by conducting catalytic reactions from 45°C to 70°C. As shown in Fig. 3, the lower optimal temperature of the immobilized κ-carrageenase than the free enzyme was attributed to the changes in physico-chemical properties induced by immobilization. κ-Carrageenase activity did not improve after immobilization because the diffusional limitations increased after covalent attachment of the enzyme to the support matrix [22,23]. More than 80% activity of the immobilized enzyme was maintained at 50 to 65°C. However, the activity of the free enzyme was sharply changed within the same temperature range (Fig. 3). The immobilized κ-carrageenase exhibited significantly lower activity stability than free κ-carrageenase (Fig. 3). This phenomenon could be explained by the fact that the restricted conformational mobility of the molecules could be obtained after immobilization [18].

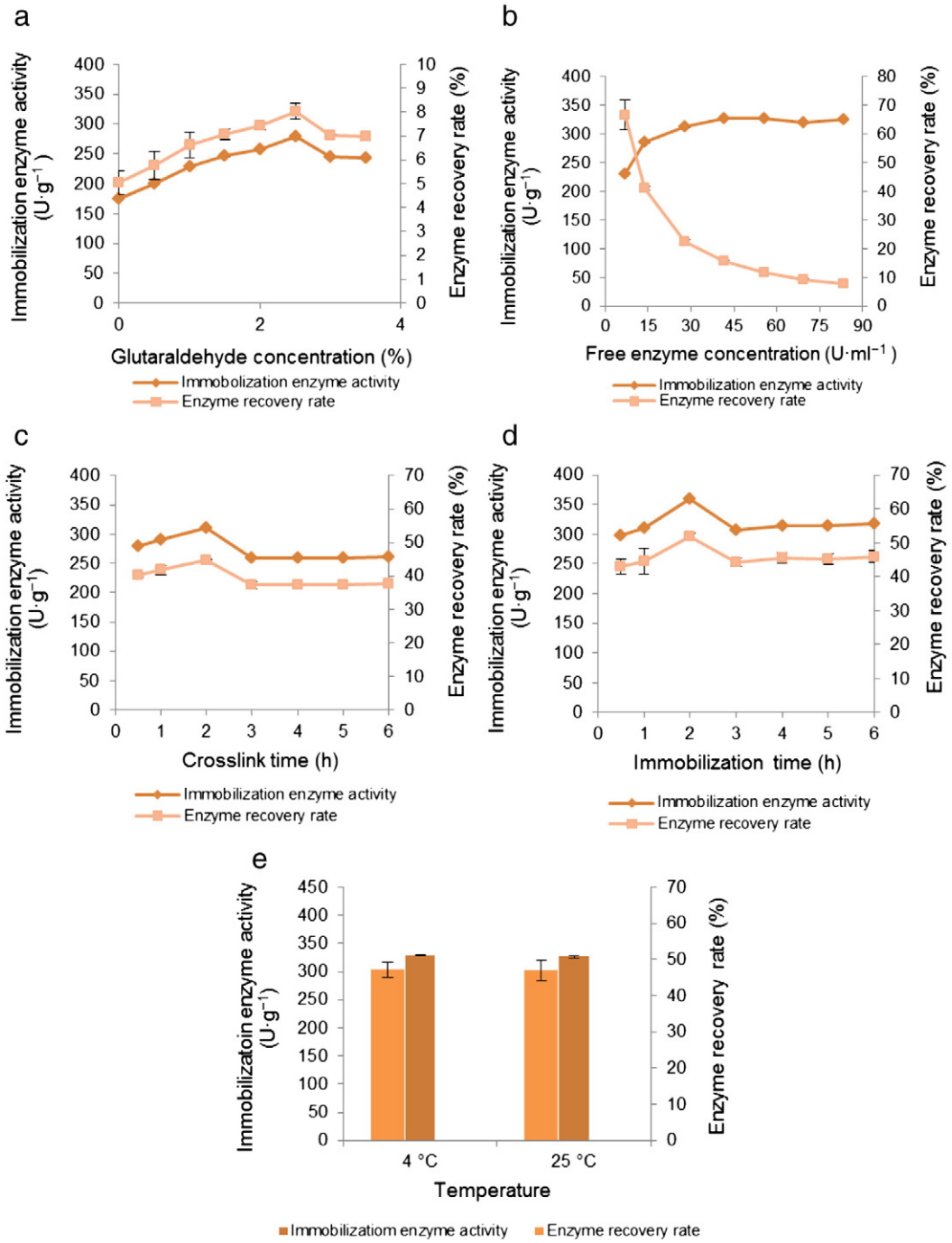

Fig. 2. Effects of glutaraldehyde concentration (a), κ-carrageenase concentration (b), cross-linking time (c), immobilization time (d), and immobilization temperature (e) on the activity of the immobilized κ-carrageenase.

The effect of pH on κ-carrageenase activity was determined under varied pH values (pH 4.0–10.0) in different buffers, and the results are presented in Fig. 4. The optimum pH value for the free and immobilized κ-carrageenase activities was 7.5. This phenomenon is also observed in another research [17]. The optimum pH of acetyl xylan esterase immobilization in the study of Saravanakumar et al. [16] is identical to that of the free enzyme. More than 80% activity was also observed in the pH range of 5.0–10.0 for the immobilized κ-carrageenase (Fig. 4), and the pH stability of the immobilized κ-carrageenase was apparently better than that of the free κ-carrageenase. This finding can be attributed to the enzyme protection by the microenvironment structure and properties formed by the CMNPs. Moreover, the microenvironment may have been buffered, thus the immobilized enzyme was less affected by the pH values [22].

3.5. Reusability and storage stability

Reusability of the immobilized enzymes is an important parameter for industrial applications; immobilized enzymes lessen production cost because of their repeated, continuous, or batch uses. Baskar et al. [24] reported that α-amylase immobilized on a magnetic support exhibits 75% residual activity after five reactions. In the present study, the residual activities for κ-carrageenase immobilized on magnetic nanoparticles were 43.5% after four cycles, and the activity gradually decreased in the successive cycles (Fig. 5).

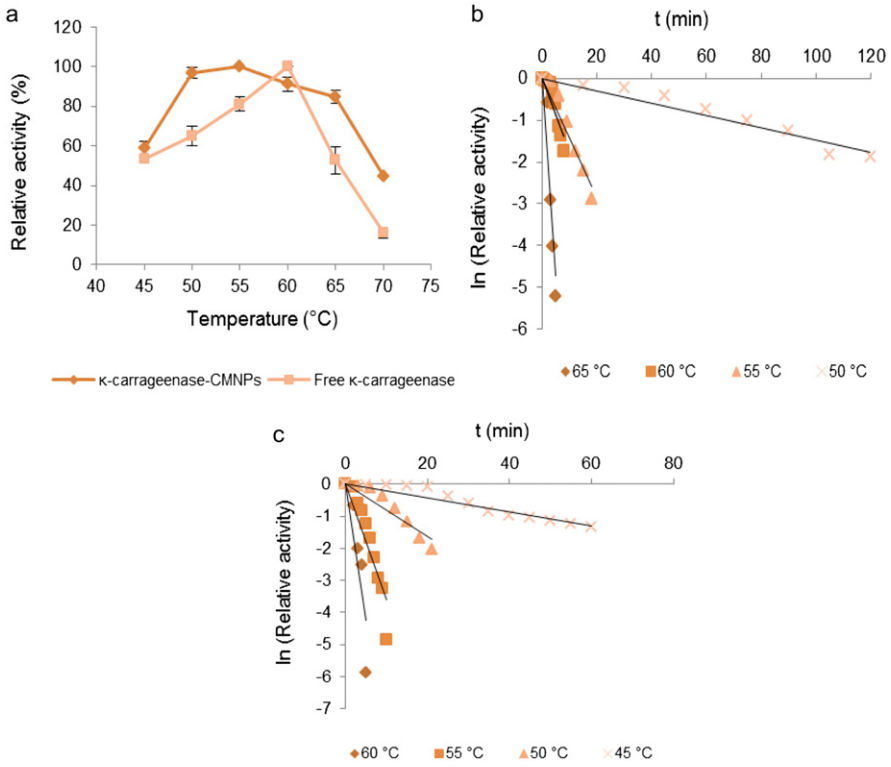

Fig. 3. Optimum temperature (a) and temperature stability (b, c) of the free (hollow icon) and immobilized (solid icon) κ-carrageenase. (Note: the actual value of 100% relative activity for immobilized and free enzyme was 410 U · g^{-1} carries and 11.3 U · mL^{-1}, respectively).

Enzyme activity is linked to the stability of its structure, and changes in this structure tend to decrease enzyme catalytic activity. As shown in Fig. 5, the half-life of free κ-carrageenase and κ-carrageenase-CMNPs at 4°C were 17.4 and 20.6 d, respectively. This result may be explained by the structure of immobilized κ-carrageenase that was stabilized as a result of the flexibility reduction of free κ-carrageenase. Therefore, multipoint covalent attachment prevents enzyme conformational changes induced by any distorting conditions [22].

3.6. Kinetic parameters

The Michaelis–Menten kinetics of the free and immobilized κ-carrageenase was investigated using a series of κ-carrageenan concentrations. The Michaelis constant (Km) was evaluated from double-reciprocal plots (Fig. 6). The calculated Km value of the immobilized κ-carrageenase was 22.5 mg · mL^{-1}, which was higher than that of free κ-carrageenase (2.28 mg · mL^{-1}). As previously reported [25,26], the increase in Km value indicated that the

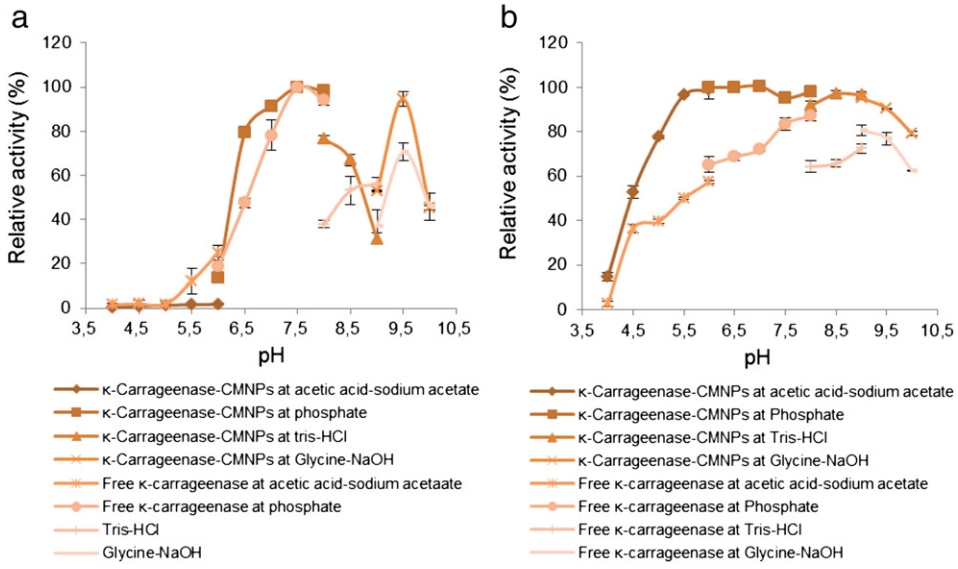

Fig. 4. Optimum pH (a) and pH stability (b) of the free and immobilized κ-carrageenase. (Note: the actual value of 100% relative activity for immobilized and free enzyme was 410 U · g^{-1} carries and 11.3 U · mL^{-1}, respectively).

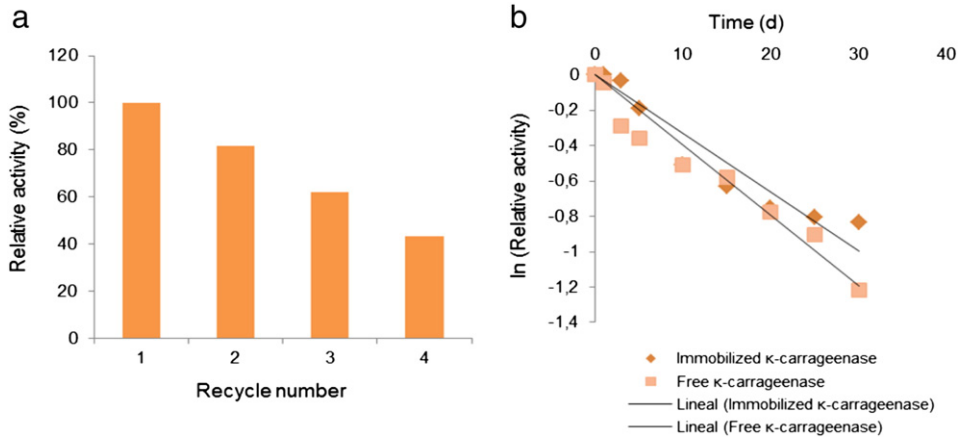

Fig. 5. Reusability of the immobilized enzyme (a) and storage stability at 4°C. (Note: the actual value of 100% relative activity for immobilized and free enzyme was 410 U · g^{-1} carries and 11.3 U · mL^{-1}, respectively).

κ-carrageenase immobilized on CMNPs had a lower binding affinity for the substrate. This finding may be due to limitation on the substrate mass transfer through the CMNPs or the steric effect that arises from the structural rigidity of the entire enzyme structure, which was distorted after covalent immobilization. Decrease of affinity to the immobilized enzyme is caused by an effect of enzymatic orientation [20].

4. Conclusions

In the present work, CMNPs were synthesized by chemical co-precipitation method and successfully immobilized with κ-carrageenase obtained from *P. carrageenovora* CICC 23819. FTIR analysis identified the bonding between κ-carrageenase and CMNPs. The optimal conditions for enzyme immobilization were 2.5% (w/v) glutaraldehyde, 13.9 U κ-carrageenase for 20 mg of magnetic nanoparticles, a 2-h cross-linking time, and a 2-h immobilization time at 25°C. Under these optimal conditions, the activity of the immobilized enzyme and enzyme recovery rate were 326.0 U · g^{-1} κ-carrageenase-CMNPs and 46.9%, respectively. Although the κ-carrageenase-CMNPs exhibited lower affinity after the immobilization process, the thermal, pH, and storage stabilities of κ-carrageenase-CMNPs were relatively higher than those of free κ-carrageenase.

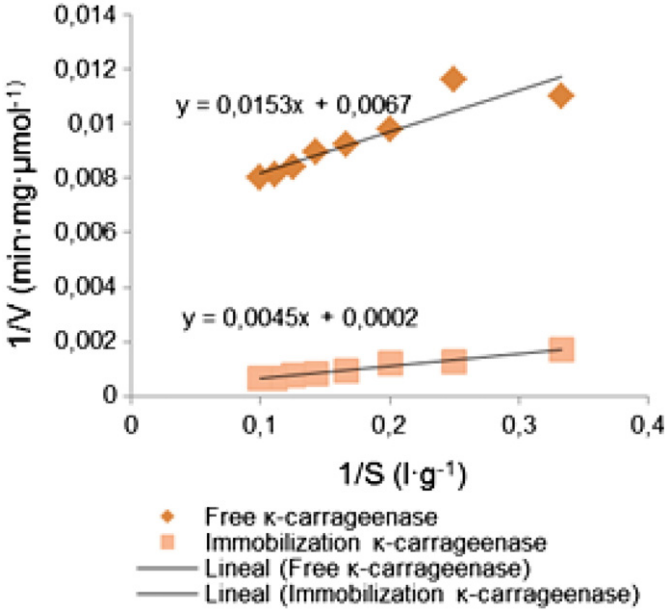

Fig. 6. Lineweaver–Burk plot of free and immobilized κ-carrageenase.

Conflict of interest

The authors declare no conflict of interest.

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