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One-pot synthesis and biochemical characterization of protease metal organic framework (protease@MOF) and its application on the hydrolysis of fish protein-waste

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ABSTRACT

A protease from *Bacillus* sp. CHA410 was purified and immobilized by a one-step MOF-embedded approach. The immobilized protease characterized using transmission electron microscopy (TEM), Fourier-transform infrared spectroscopy (FT-IR), and scanning electron microscopy (SEM). The optimal pH activity of protease CHA410 and protease@MOF was obtained at pH 8.0 and 9.0, respectively. Thermostability results after 160 min incubation showed that a 25 and 41 % enhancement in the relative activity of protease@MOF observed at 60 and 70 °C, respectively, compared to free protease. *K*m of the free and immobilized protease@MOF in the presence of casein was 0.685 and 0.033 mg/mL, respectively. Also, *K*m of the free and immobilized protease@MOF in the presence of fibrin was 0.292 and 0.145 mg/mL, respectively. The Fibrinolytic activity/Caseinolytic activity ratio (F/C ratio) of the free and immobilized proteases was 0.36 and 0.43, respectively. Protease activity of both forms of the enzyme was increased in the presence of some divalent cations, including Ca^{2+}, Mn^{2+}, Mg^{2+}, and Zn^{2+} ions, while it intensely diminished by phenylmethylsulfonyl fluoride (PMSF), proposed as serine-protease. A 10 and 82 % enhancement in protease activity of free and immobilized proteases was achieved in the presence of butanol, respectively. Storage stability results showed that the immobilized enzyme retained about 70 % of its original activity at the end of this period, while the free enzyme only showed 22 % of its initial activity. The hydrolysis degree of immobilized protease CHA410 in the hydrolysis of fish protein waste was obtained about 46 % after 2 h of incubation at 50 °C. In comparison, it was gained about 20 % for protease CHA410 at a similar situation. Finally, results indicated that the free and immobilized protease could be used in the food industry for the hydrolysis of fish protein waste.

1. Introduction

Microbial proteases have several applications in industrial and also academic researches. They found one of the financially significant enzyme groups. These enzymes are commonly used in thrombolytic treatment, peptide synthesis, protein hydrolysates production, photographic, textile, and bioremediation [1]. Because of extensive biochemical diversity, low-cost production, and dissolving fibrin clots directly, the fibrinolytic proteases are commonly used as thrombolytic agents in treating cardiovascular diseases [2,3].

Furthermore, protein hydrolysates are high-energy complements and hypoallergenic foods. The enzymatic production of these compounds concretes an approach for growing the nutritional possessions of the hydrolysates [4,5]. Recently, hydrolyzed proteins from several origins, including rice bran [6], canola [7], sardinelle (*Sardinella aurita*) [8], Sardinelle (*Sardinella aurita*) muscle proteins [9], golden apple snail (*Pomacea canaliculata*) [10], and sacha inchi [11] have been established to keep the antioxidant ability.

Finding new enzymes with proper stability and specificity to temperature, pH, metallic ions, surfactants, and organic solvents has been appreciated for the researchers. Bacterial proteases have been isolated from different sources, including *Bacillus*, *Shewanella*, *Yersinia*, *Pseudomonas*, *Alteromonas*, and *Flavobacterium* [12–16]. *Bacillus* proteases have been proven as the best commercial proteases among all of the bacterial proteases [17].

However, free enzymes showed several drawbacks, including the short catalytic lifespan, inadequate reusability, and low thermal stability. Enzyme immobilization is an efficient method to overcome these disadvantages. Facile separation from the product, diminish downstream product processing, reduce the cost of production, increase

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operational stability are some of the advantages of enzyme immobilization [18,19]. Numerous materials have been used for enzyme immobilization. Due to a large surface to volume ratio of nanomaterials, they have been suggested as innovative materials for the enzyme immobilization. Different nanomaterials, such as magnetic nanoparticles, silica, carbon nanotubes, electrospun nanofibers, and polymer beads, have been used for enzyme immobilization [20].

Metal-organic framework (MOF) are hybrid porous materials making from metal ions and organic ligands connected by tight linkages. They showed more excellent features such as high surface region, large void volume, huge porosity, mild production environments, the facile tenability of pore size, and facile adjustable surfaces [21–23]. One-pot synthesis of enzyme-embedded MOFs was achieved by Lyu et al. 2014 [24]. In this approach, the enzyme was straight embedded through MOF crystal construction, in which the enzyme immobilized in MOFs revealed high catalytic efficiency against the free enzyme [25]. For example, glucose oxidase- immobilized in mZIF-8 [26], glucoamylase immobilized into ZIF-8 [27], and lipase embedded in ZIF-8 [28,29]. Furthermore, enzymes embedded in MOF have performed for several enzymes, including lipase [32], laccase [33], urease [34], glucose amylase [28]. Despite this, there are just a few reports about protease-embedded MOF. This is the first report about the immobilization of a fibrinolytic protease by the in situ MOF approach [35].

Due to some fascinating outcomes of ZIF-8, including minor cytotoxicity, higher chemical stability, and facile synthesis in alcohol or water solution [30,31], it is a fantastic approach for enzyme immobilization. In the present work, a fibrinolytic protease from *Bacillus* CHA410 isolate has been purified and characterized. Then this enzyme embedded in ZIF-8, morphologically, and biochemically characterized. Finally, the ability of both forms of the enzyme in the hydrolysis of the protein fish-waste has been considered.

2. Experimental

2.1. Materials

Zinc nitrate, 2-methylimidazole, Na_2HPO4, NaCl, and KH_2PO4 were purchased from Merck. Fibrin, casein, and albumin were acquired from Sigma-Aldrich. All the other chemical substances were in analytical grade. Casein, agar, yeast extract, peptone, nutrient agar, nutrient broth, $KH_2PO_4{\cdot}6H_2O$, $K_2HPO_4{\cdot}6H_2O$, $MgSO_4{\cdot}H_2O$, and other salts purchased from Merck company. Thrombin, urokinase, fibrinogen (human), and albumin (bovine) were acquired from Sigma-Aldrich. Trichloroacetic acid (TCA) was purchased from Scharlau Company. All nanocomposites were characterized by a scanning electron microscope (SEM, Philips XL 30) with gold coating. Transmission electron microscopy (TEM) images were determined on a Tecnai-G20 (FEI, USA) transmission electron microscope. The Fourier-transform infrared (FTIR) spectroscopy spectra of all nanocomposites have been considered between 4000 and 500 cm^{-1} as KBr pellets on SHIMADZU FT-IR 8400 Spectrometer (Kyoto, Japan). The absorption data were obtained using UV–vis spectrophotometer (Carry 50).

2.2. Isolation and identification of bacterial protease

Soil and water samples isolated from Dimand hot spring located in Jiroft (Iran). Protease producing bacteria screened in the culture containing peptone (1.0 % w/v), yeast extract (10 % w/v), NH_4Cl (4 % w/v), and $CaCO_3$ (0.08 % w/v) for two days at 37 °C, 150 rpm [3,36]. After that, the culture was streaked on the casein, skimmed milk, and fibrin agar media and maintained at 37 °C for two days. The CHA410 isolate, which showed the highest halo, was chosen for further studies.

Molecular identification of bacterial isolate was done by isolating genomic DNA (using genomic DNA isolation kit (Bioneer, South Korea) and PCR amplification of the 16S rDNA gene using specific primers. Forward primer 8 F: 5′-AGAGTTTGATCMTGG-3′ and 1492 R: 5′-ACC TTGTTACGACTT-3′ reverse primer were used in this study. PCR was done according to the following method composed of 4 min at 94 °C for primary denaturation and 30 cycles consisting of 30 s at 94 °C, 30 s at 55 °C, and 45 s at 72 °C and finishing extension at 72 °C for 10 min. The comparison of the obtained gene sequence with the other sequences was accomplished by BLAST through the National Center of Biotechnology Information server with an accession number of MT355498. The phylogenetic tree of this isolate created using the neighbor-joining method in the MEGA X software tool. Morphological and biochemical identification of this isolate was considered by Bergey's Manual of Determinative Bacteriology [37].

2.3. Production and purification of fibrinolytic protease CHA410

Enzyme production was performed in the culture having peptone (1.0 % w/v), yeast extract (1.0 % w/v), NH_4Cl (4% w/v), and $CaCO_3$ (0.08 % w/v) complemented with 2 % (w/v) casein, pH 7.0, for two days at 37 °C, 150 rpm [3,36]. For pre-culture medium, CHA410 isolate was cultured in Nutrient broth medium and incubated overnight at 37 °C. After that, 1 % (v/v) of the pre-culture medium was inoculated in the production medium and maintained for 48 h at 37 °C. The cell-free extract was obtained by culture centrifugation at 8000 × g for 20 min at 4 °C.

The crude enzyme was concentrated by ammonium sulfate (85 %) and then maintained at 4 °C overnight to precipitate. The proteins precipitate were gathered by centrifugation at 12,000 × g for 20 min at 4 °C. The obtained precipitates were suspended in Tris-HCl buffer (50 mM, pH 8.0) and dialyzed toward a similar buffer. After each 8 h of incubation, the buffer was changed with the same buffer three times. Finally, the dialyzed protein was laden to the Q-Sepharose column (10 × 1 cm), which pre-equilibrated with Tris-HCl buffer (50 mM, pH 8.0). The bound enzymes were eluted from the column with NaCl gradient (0.1–0.5 M), and active fractions were gathered and assayed for the enzymatic activity. The molecular weight of the protease BC1 was considered by SDS-Page (12 %), as reported by Laemmli [38]. The protein concentration of active fractions was measured by the Bradford technique [39], while bovine serum albumin (BSA) was used as the standard.

2.4. Fibrinolytic protease assay

Enzyme activity of protease CHA410 was considered according to the previous method with minor revision [40,41]. Typically, 100 µL enzyme (free/immobilized) mixed with 300 µL Tris/HCl buffer (0.5 M, pH 8.0) containing 100 mM calcium chloride. Then, 100 µL of fibrin (1.0 %, w/v) was mixed and maintained at 50 °C for 15 min. After that, the enzyme activity was stopped by adding 500 µL of trichloroacetic acid (10 %). After centrifugation at 10,000 × g for 8 min, the absorbance of obtained supernatant was considered at 280 nm, and the fibrinolytic activity was measured [40]. The enzyme unit was measured by increasing the absorbance, which is corresponding to 1 µg of tyrosine/min. The caseinolytic activity was also measured at the same procedure with some modifications. Instead of fibrin, casein was used with the same volume and concentration [3].

2.5. Synthesis of protease@MOF

For the production of the protease@MOF nanocomposite, $Zn(NO3)_2$ (1 mM) was mixed with protease CHA410 (0.02 $mgmL^{-1}$) and 2-methylimidazole (1 mM). The solution was mixed for about 60 min. After incubation at room temperature overnight, the pellet was gathered by centrifugation at 8000 rpm for 10 min and washed with de-ionized water three times. Supernatants were gathered to calculate the protease amount using the Bradford technique [39]. The pellets were dried in a vacuum dryer and kept on 4 °C for future use. The activity recovery

(AR) was calculated by Eq. (1): AR (%) = whole specific activity of protease@MOF/primary specific activity of protease CHA410 × 100 (1). Encapsulation yield (EY) was calculated by Eq. (2): EY (%) = initial protein concentration-protein concentration in supernatant/initial protein concentration × 100.

2.6. Biochemical characterization of free and immobilized protease

The enzyme activities of both forms of proteases were also considered at diverse pH ranges. Various buffers (50 mM), including sodium acetate buffer (pH 4.0–6.0), sodium phosphate buffer (pH 7.0–8.0), Tris/base buffer (9.0–10.0), and glycine buffer (11.0–12.0) were used in this study. The relative activities were considered, as formerly defined in Section 2.4. The temperature activity profiles of both forms of enzyme were examined at 20–90 °C. The relative activities were considered, as formerly defined in Section 2.4. The Michaelis–Menten, and Lineweaver–Burk plots of free and immobilized proteases (2 mg) were considered at subsequent substrate concentrations (0.0–0.6 mM) at 50 °C. Kinetic parameters, including K_m, k_{cat}, and K_{cat}/K_m for the free and immobilized proteases, were also determined at different concentrations of fibrin and casein.

2.7. Tolerance to organic solvents

Free and immobilized proteases, having the same concentration of lipase, were incubated in 2 mL of organic solvents (50 % v v^{-1}) for 120 min. Then the free enzyme and protease@MOF isolated by centrifugation at 8000 rpm for 8 min and washed three times with PBS buffer (10 mM, pH 8.0). The stabilities of the immobilized protease to these organic solvents were investigated against the free enzyme.

2.8. Effect of metal ions and protease inhibitors on enzyme activity

The effect of some metal ions, including Na^{+1}, K^{+1}, Ca^{+2}, Mg^{+2}, Ba^{+2}, Cu^{+2}, Zn^{+2}, Mn^{+2}, Hg^{+2}, Al^{+3}, and Fe^{+3} was considered on the protease activity of both forms of the enzyme under optimal condition. The effect of some protease inhibitors such as ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), 110-phenanthroline, and iodoacetic acid was also considered on the protease activity. These enzymes were pre-incubated with the mentioned metal ions and protease inhibitors (2 mM) for 2 h in the phosphate buffer (20 mM, pH 8.0). The protease activity in the absence of any metal ions/inhibitors was considered as 100 %.

2.9. Thermal, storage stability and reusability

The thermal stability of both forms of protease was examined by incubating an equivalent quantity of these enzymes under optimum situations in PBS buffer solution (50 mM, pH 8.0) at 60 and 70 °C. The remaining activity of these enzymes was calculated at various time intervals, while the original activity was deliberated as 100 %. The storage (4 °C) stability of both forms proteases was also considered.

The reusability of the immobilized protease was evaluated after repeated cycles. After each cycle, the enzyme nanocomposites were centrifuged at 8000 rpm for 8 min and washed three times with PBS buffer (10 mM, pH 8.0). Then, the relative protease activity (%) was considered as the ratio of the residual protease activity to the primary protease activity. The leaching of the protein from these nanocomposites was determined by Eq. (3): Leaching (%) = (protein concentration in supernatants/initial protein concentration) × 100.

2.10. Proteolytic activity with different substrates

Enzyme activity of free and immobilized fibrinolytic BC1 was considered in the presence of different substrates (1 % w/v), including fibrin, hemoglobin, casein, transferrin, and albumin under the optimal condition. Enzyme activity of both forms of enzyme in the presence of fibrin was considered as 100 %. One unit of enzyme activity was considered as releasing of 1 μg of tyrosine/min at 37 °C. Furthermore, the ratio of fibrinolytic activity/caseinolytic activity (F/C ratio) was also measured.

2.11. Preparation of protein hydrolysate of Thunnus tonggol waste

Protein hydrolysate of *Thunnus tonggol* waste was isolated by the method, which formerly reported by Jemil et al. [2014] [42]. Briefly, 0.4 L of distilled water was added to 200 *g* of fish muscle, homogenized, and heated for 0.5 h at 100 °C to deactivate the endogenous proteases. The resulting fillets were dried at 80 °C for 20 h and powdered. The activity of free and immobilized proteases was investigated in the presence of the different concentrations of the protein isolated under optimal conditions [41]. Furthermore, time-dependent protein waste hydrolysis by free and immobilized proteases was also investigated. The pH of the reaction was preserved at the optimal level by continuous adding of 4 N NaOH. After different time intervals (0, 0.5, 1.0, 2.0, 4.0, 8.0, and 12 h), the enzymes were inactivated by the addition of HCl solution (0.1 N) [9]. Soluble fractions were isolated by centrifugation at 6000 × g for 15 min, and protein concentrations were determined by the Bradford method [39]. The degree of hydrolysis (DH) was also measured as follows [41,44]:

3. Results and discussion

3.1. Fibrinolytic protease production and purification

The CHA410 isolate showed the 44 and 30.1 mm clear halo on the casein and fibrin agar plates, respectively. The culture supernatant established the highest F/C ratio among the bacterial strains signifying that the CHA410 isolate is a potent fibrinolytic producing bacterium. The sequence homology of the 16S rDNA gene of the CHA410 strain showed that this isolate has extreme homology with *Bacillus subtillus* (97 %). The phylogenetic tree of this isolate was created using the neighbor-joining method in the MEGA X software tool. The crude extract of CHA410 isolate was performed to ammonium sulfate precipitation (85 % saturation). Protein pellet centrifuged, dialyzed, and played onto Q-Sepharose chromatography. Results showed that fibrinolytic CHA410 isolate was purified with 28-fold purification and 18.1 % yield. Choi and co-workers reported that the purification yield and fold were 0.9 % and 20.71-fold, respectively [45]. Mahajan and coworkers purified a fibrinolytic enzyme from *B. subtilis* ICTF-1, with purification fold of 32.42 and 7.5 % recovery [46]. SDS–PAGE results showed that the fibrinolytic CHA410 has a single band of about 33 kDa. It was similar to the other *Bacillus* proteases (20–44 kDa). The molecular mass of some fibrinolytic enzymes was reported as *B. subtilis* KCK-7 (44 kDa), *Bacillus subtilis* C142 (23.5 kDa) [45], and *B. subtilis* QK02 (42 kDa) [47]. It was also different from serine proteases from *Bacillus* sp. DJ-4 (29 kDa) [48], *Streptomyces* sp. (30 kDa) [49], and *B. subtilis* ICTF-1 (28.0 kDa) [46].

3.2. Fibrinolytic CHA410 immobilization and characterization

Determination of protein concentration by the Bradford technique presented that protein embedded in MOF was attained effectively. Furthermore, results showed that the efficiency of immobilization on this MOF was achieved by about 77 %; given the mass of MOF, the total enzyme immobilized is low. Junior and co-workers reported that the immobilization efficiency of collagenase was obtained about 85 % for integration in ZIF-8 during its biomimetic mineralization [49]. Nadar and Rathod reported that the encapsulation yield of glucoamylase was about 99 %, with relative activity of 118 U/mg [28]. In our previous study, we constructed the CLEA of a protease on functionalized magnetic nanoparticles, and results showed that 70 % of protein loading

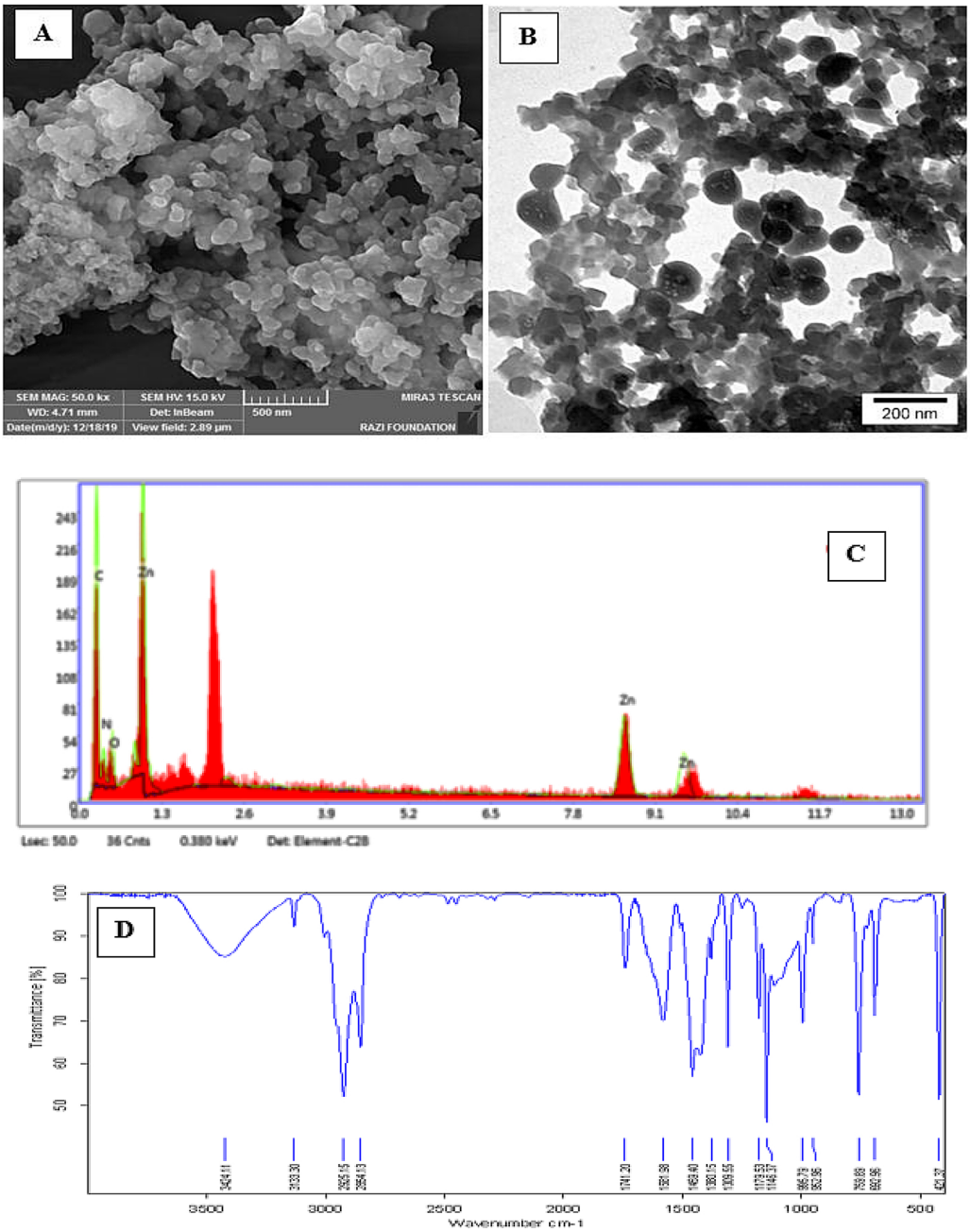

Fig. 1. SEM image of protease@MOF (a), TEM image of protease@MOF (b), Edax of protease@MOF (c), and FTIR analysis of protease@MOF (d).

was achieved in that approach [50].

3.3. Analytical characterization

Because of significant enduring porosity and high chemical and thermal tolerance, Zeolitic imidazolate frameworks (ZIFs) fascinate great attention. The immobilized fibrinolytic CHA410 was analyzed by FTIR, FESEM, TEM, and EDX. The surface morphology of produced protease-MOF showed the homogeneous size distribution and an average size of 65 nm by scanning electron microscope (SEM) and transmission electron microscope (TEM) (Fig. 2a, b). The hexagonal prism structured morphology was reported in the previous papers [22,28]. EDS analysis displayed that Zn elements were detected in protease@MOF (Fig. 1c) [28,34].

FTIR spectra of protease@MOF showed that the vibrational peaks in the range of 600–1500 cm^{-1} resemble the bending and stretching of the imidazole ring (Fig. 1d). Moreover, the 1581 cm^{-1} peak is related to the stretching of C—N bonds in 2-methyl imidazole. The peaks at 2925 and 3133 cm^{-1} could be recognized to the vibration of C—H bonds of the aliphatic chain and the aromatic ring of 2-methyl imidazole, respectively [27,51]. These peaks were proved that the ZIF-8 structure does not change after washing [52]. Rafiei and co-workers showed that the free enzyme displayed a peak at 1660 cm^{-1}, representing the CO stretching of amide I bond, while the peak at 3406 cm^{-1} characterized by the CO stretching manner of NH stretching [53]. These bands were approving the existence of the enzyme in ZIF-8 (Fig. 1d).

3.4. Biochemical characterization of free and immobilized proteases

3.4.1. Effect of pH and temperature on the fibrinolytic activity

pH-dependent fibrinolytic activity of free and immobilized protease@MOF was considered. Free fibrinolytic CHA410 showed the

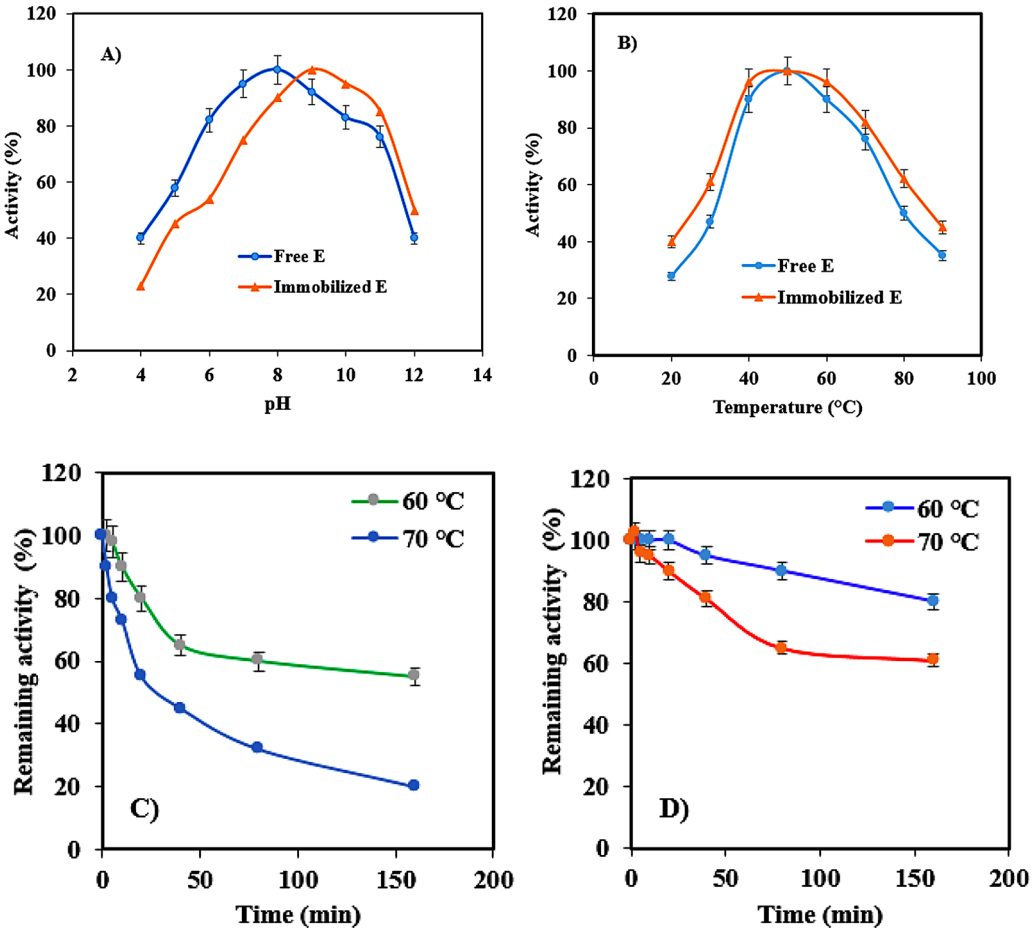

Fig. 2. pH (a) and temperature (b) activity profiles of free and immobilized fibrinolytic protease CHA410. Thermal stability of free (c) and immobilized (d) fibrinolytic protease CHA410 in the different time intervals.

maximal activity at pH 8.0 (Fig. 2a). Previously reports indicated that the fibrinolytic serine proteases are usually active at a pH range of neutral and alkaline conditions, in which the optimal pH found between 8.0–10 [2]. Mahajan et al. 2011 reported that the maximal pH activity of fibrinolytic protease from *Bacillus subtilis* ICTF-1 obtained at pH 9.0 [46]. Choi et al. 2017 reported that the protease C142 was active at weak acidic and alkaline conditions, which maximal activity obtained at pH 6.0 [45]. Overall, fibrinolytic proteases had an optimum pH between 6.0–7.0, except fibrinolytic protease from *R. chinensis* 12 showed the optimum activity at pH 10.5 [54].

The pH activity profile of the protease@MOF showed the maximum activity at pH 9.0. Furthermore, a 28 and 12 % enhancement in the fibrinolytic activity of the protease@MOF observed at pH 6.0 and 10.0, respectively, compared to the free enzyme (Fig. 2a). It might be related to the electrostatic interactions between the MOF components and solution, causing the inadequate dividing of H^+ and OH– in microenvironment and substance solution [55,56].

The influence of temperature on the activity of both forms of protease was also investigated at various temperatures (Fig. 2b). Results exhibited that both types of enzyme showed maximum activity at 50 °C. It is the same as the optimum activity of fibrinolytic protease from *B. subtilis* ICTF-1 (50 °C) and higher than the protease C142 (40 °C) [45,46]. Tightly bound of nitrile hydrolase in NHase@Co-Cys structure caused a shift in optimum temperature and extended temperature activity range [57], which promoted the catalysis activity at an evaluated temperature [58]. Additionally, Wang and co-workers indicated that the different conformational changes and structural rigidification might be caused by the inactivation of NHase@Co-Cys [56].

3.4.2. Thermal tolerance of the free and immobilized proteases

The thermal tolerance of both forms of fibrinolytic proteases was examined by incubating them at 60 and 70 °C. Results showed that the protease@MOF showed better thermal tolerance than the free protease. Moreover, after 160 min incubation, a 25 and 41 % enhancement in the relative activity of the protease@MOF obtained at 60 and 70 °C, respectively, compared to the free enzyme (Fig. 2c, d).

The purified *B. subtilis* ICTF-1 protease showed high stability up to 37 °C and became unstable above 37 °C [46]. Furthermore, *B. subtilis* ICTF-1 protease showed 50 and 18 % of its original activity after 1 h incubation at 40 and 50 °C, respectively, but it completely deactivated after 10 min incubation at 60 and 70 °C [46].

It is possibly related to the restriction of the conformational movement of the protease@MOF at different temperatures due to the several covalent interactions between the enzyme/enzyme and enzyme/supports, which keeps it from denaturation [59]. Hence, the free enzyme could simply be denaturized, while the protease@MOF is sheltered because of rigid conformation, and could preserve its activity [60].

3.4.3. The kinetic parameters of free and immobilized proteases

The kinetic parameters of both forms of protease were obtained from the double reciprocal plot in different concentrations of fibrin and casein (Fig. 3). *K*m of the free protease and protease@MOF in the presence of casein was 0.685 and 0.033 mg/mL, respectively. Also, *K*m value of the free protease and protease@MOF in the presence of fibrin was 0.292 and 0.145 mg/mL, respectively (Table 1). Results showed that the *K*m values of the immobilized fibrinolytic enzyme are 20.75 and 2 folds lower than the free enzyme in casein and fibrin,

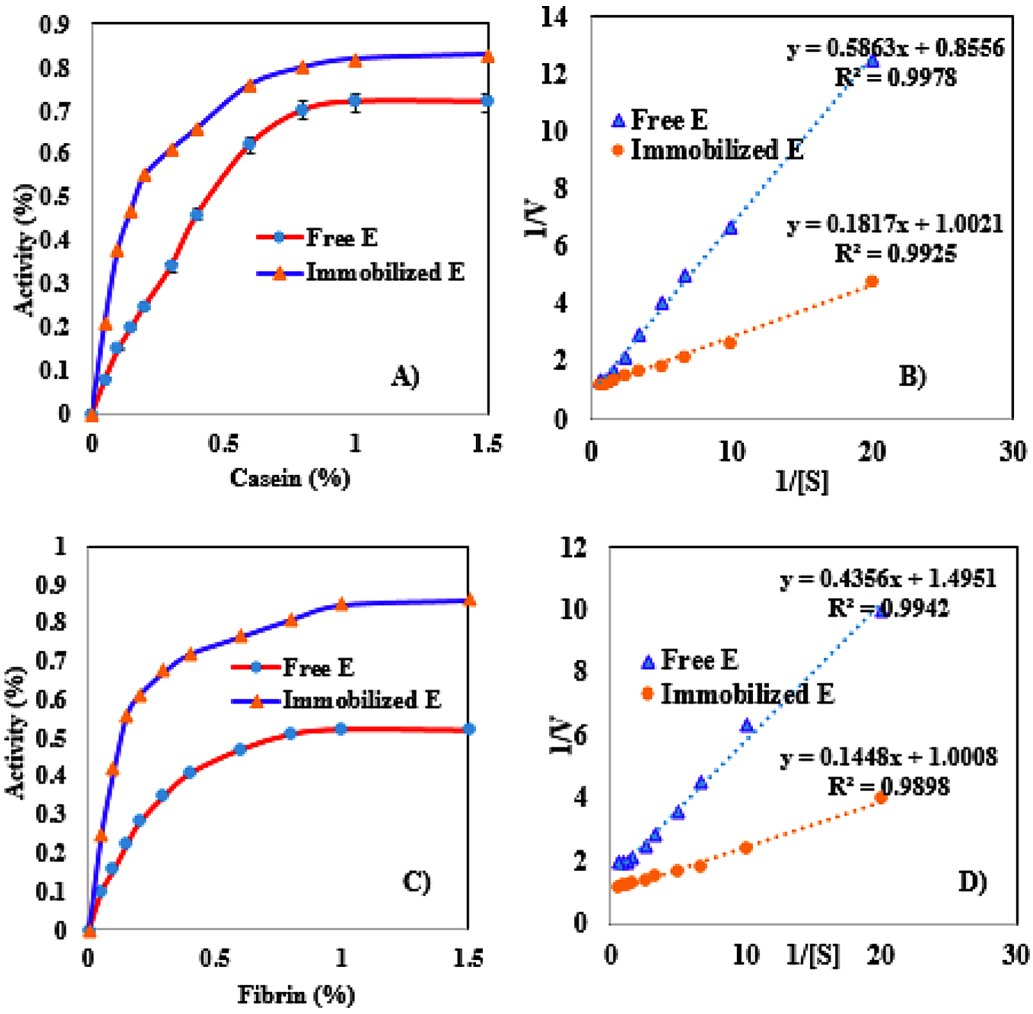

Fig. 3. Michaelis–Menten (a) and Lineweaver–Burk (b) plots of free and immobilized fibrinolytic protease CHA410 in the presence of different concentrations of casein. Michaelis–Menten (c) and Lineweaver–Burk (d) plots of free and immobilized fibrinolytic protease CHA410 in the presence of different concentrations of fibrin.

Table 1
kinetic parameters of free and immobilized protease CHA41 in the presence of different concentration of casein and fibrin.

	*K*m (mg/mL)	*Vmax (mg/mL/min)*	*K*cat (1/min)	*K*cat/Km (min/mg/mL)	Substrate
Free CHA41	0.685	1.169	22.21	32.42	Casein
Immobilized E	0.033	0.997	18.94	573.94	Casein
Free CHA41	0.292	0.669	12.71	43.53	Fibrin
Immobilized E	0.145	0.999	18.98	130.9	Fibrin

respectively.

The decrease in the K_m value of protease@MOF was due to exposure of active centers caused by in situ biomineralization procedure. Thereby, it led to an improvement in the affinity of the enzyme towards the substrate. The same results were also reported by the other immobilized enzymes in the MOF structure [24,27,28,61,62]. Wang and co-workers reported that the *K*m value of enzyme@MOF was 1.82 fold lower than the free protease [56]. Furthermore, Virendra and Rathod immobilized a proline activated lipase within metal organic framework. They showed that the immobilized enzyme showed less *K*m value than the free enzyme. They suggested that the biomineralization procedures afford highly ordered MOF superstructures around the enzyme molecules to maintain the catalytically active tertiary molecular structure of enzyme [61].

Besides, the Vmax value of protease@MOF was 1.5 fold higher than the free enzyme in the presence of fibrin, which might be related to the molecular crowding of substrates in the permeable structures of enzyme@MOF [56]. The *K*m values of fibrinolytic enzymes reported about 0.66 mg/mL [3], 4.2 mg/mL [63], 0.96 mg/mL [64], 1.216 mg/mL [65], and 0.09 – 0.4 g/mL [66], using casein/azocasein as substrate.

These results are in agreement with the *K*m of the immobilized serratiopeptidase on magnetic nanoparticles [67]. Li and co-workers constructed the enzyme-CuBDC nanocomposites using *Candida rugosa* lipase, horseradish peroxidase, and trypsin. Their results exhibited that due to limited mass transfer or enzyme denaturation by CuBDC, the enzyme activity decrease [68].

3.4.4. Protease activity in the presence of protein substrates

The proteolytic activity of both forms of protease was considered in the presence of different protein substrates (1 % w/v), including transferrin, hemoglobin, albumin, ɣ-globulin, and casein. Protease activity in the presence of fibrin was measured as 100 %. Results showed that the free and immobilized proteases showed 2.44 and 4.05 folds higher activity than the control toward albumin, respectively. Fibrinolytic activity/Caseinolytic activity ratio (F/C ratio) of free and immobilized proteases was 0.36 and 0.43, respectively. This F/C ratio was higher than that of the other bacterial proteases from *Serratia marcescens* subsp. *Sakuensis, Streptomyces griseus*, *Tritirachium album* [69], and commercially prepared protease Subtilisin BPN whereas the

Table 2
protease activity of free and immobilized CHA41 in the presence of different metal ions and protease inhibitors.

	Concentration	Relative activity* (%)	
		Free enzyme	Immobilized E
control	–	100	100
Cu^{2+}	0.5	107 ± 0.6	110 ± 0.5
Zn^{2+}	0.5	98 ± 0.2	112 ± 0.6
Co^{2+}	0.5	82 ± 0.1	87 ± 0.4
Li^{2+}	0.5	83 ± 0.3	92 ± 0.2
Mn^{2+}	0.5	77 ± 0.5	82 ± 0.2
Ca^{2+}	0.5	122 ± 0.4	138 ± 0.5
Mg^{2+}	0.5	82 ± 0.2	98 ± 0.2
Fe^{2+}	0.5	81 ± 0.3	94 ± 0.1
Hg^{2+}	0.5	85 ± 0.1	90 ± 0.3
Na^{+}	5	130 ± 0.2	144 ± 0.4
K^{+}	5	87 ± 0.3	93 ± 0.2
EDTA	5	62 ± 0.1	71 ± 0.1
PMSF	5	17 ± 0.1	25 ± 0.1

* Data represents mean ± SD (n = 3).

human plasmin demonstrated the maximal F/C ratio [70].

However, results showed no proteolytic activity was observed in the presence of the plasma proteins, including γ-globulins, hemoglobin, and transferrin. These results may be related to the multipart nature of these substrates, which prevent the hydrolysis of the protein substrates and is favorable for human use as a therapeutic agent.

Krishnamurthy and Belur, 2018 were also done the same tests for the fibrinolytic protease from *Bacillus megaterium* KSK-07 [3]. Their results showed a slight enzyme activity in the presence of some blood proteins such as elastin, mucin, hemoglobin, γ-globulins, and collagen [3].

3.4.5. Effect of metal ions and chemical compounds on the fibrinolytic activity

The effects of some protease inhibitors and metal ions on the protease activity of free and immobilized fibrinolytic proteases were considered (Table 2). Results showed that the activity of both forms of enzyme decreased in the presence of phenylmethyl-sulphonyl fluoride (PMSF) and ethylene diamine tetra acetic acid (EDTA) as compared to the control, signifying that this enzyme belongs to the serine metalloprotease family similar to the previous reports [45,63,71].

The enzymatic activity of proteases is mainly influenced by metal ions, which as activator or inhibitor. Generally, metal ions act as electrophiles that hold the functional groups in three-dimensional orientations, and they also form enzyme–substrate interaction by coordinate bond formation. Apart from this, metal ions also stabilize the catalytic active site of enzymes [72]. Therefore, metal ions in this regard plays an essential part in the activation and stabilization of enzymes [73].

The protease activity of both forms of the enzyme was considered in the presence of some metal ions (5 mM), including K^{+}, Na^{+}, Co^{2+}, Cu^{2+}, Pb^{2+}, Li^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, Fe^{3+}, Fe^{2+} at optimal condition. As a control, protease activity without any metal ions was considered as 100 %. Similar to the results of Yogesh and Halami [71] and Peng and co-workers [2], the activity of both free and immobilized proteases was increased in the presence of some divalent cations, including Mg^{2+}, Ca^{2+}, Mn^{2+}, and Zn^{2+} ions (Table 2). It is agreed to the previous reports, which indicated that these enzymes need divalent metal ions [2]. Furthermore, the activity of free and immobilized proteases increased about 30 and 44 % in the presence of Na^{+}, respectively, while it decreased about 13 and 7 % in the presence of K^{+}, respectively (Table 2). The same results were also reported by C142 protease, in which Na^{+} increase protease activity by about 129 % [49].

Table 3
protease activity of free and immobilized CHA41 against several detergents and organic solvents.

	Concentration (%)	Relative activity* (%)	
		Free enzyme	Immobilized E
control	–	100	100
SDS	1	66 ± 0.1	76 ± 0.2
CTAB	1	74 ± 0.2	85 ± 0.1
Tween 20	1	97 ± 0.2	99 ± 0.4
Tween 80	1	96 ± 0.1	97 ± 0.4
Triton X-100	1	105 ± 0.4	110 ± 0.3
H_2O_2	1	87 ± 0.1	93 ± 0.2
Ethanol	50	105 ± 0.5	116 ± 0.4
Methanol	50	102 ± 0.3	112 ± 0.1
Butanol	50	110 ± 0.4	182 ± 0.6
n-hexane	50	85 ± 0.2	96 ± 0.1
Isopropanol	50	87 ± 0.3	110 ± 0.3
Chloroform	50	84 ± 0.2	97 ± 0.5
DMSO	50	108 ± 0.6	118 ± 0.6
DMF	50	81 ± 0.5	92 ± 0.4

* Data represents mean ± SD (n = 3).

3.4.6. Effect of some organic solvents and surfactants on protease activity

The protease activity of both forms of the enzyme was investigated in the presence of some surfactants, including SDS, CTAB, Tween 20, Tween 80, Triton X-100, and H_2O_2. As shown in Table 3, both forms of the enzyme showed high stability in the presence of Tween 20, Tween 80, and Triton X-100. The activity of free enzyme decreased about 34, 26, and 13 % in the presence of SDS, CTAB, and H_2O_2, respectively, while the activity of immobilized enzyme decreased about 24, 15, and 7 %, respectively, at the same condition. The same results were also reported by a protease from the *Serratia marcescens* [3].

The impact of different organic solvents on the stability of both forms of protease CHA410 was investigated. Results showed that the protease stability of free and immobilized enzyme decreased in the presence of DMF and chloroform, but increased in the presence of butanol and DMSO. A 10 and 82 % enhancement in protease activity of both forms of enzymes was achieved in the presence of butanol, respectively (Table 3).

Protease stability in non-aqueous solvents is due to this idea that the organic solvent reserves the protein in open and flexible conformation and causes the protease lid does not shelter the cleft of the active site [74,75]. Moreover, organic solvents change the polarity of the enzyme reaction. Due to water striping phenomena from the enzyme hydration layer by some non-aqueous solvents; the non-stable protease becomes unfolded and inactive in the presence of non-aqueous solvents [76].

3.5. Storage stability and reusability studies

The activity of the protease@MOF for 28 days with one-day intervals is revealed in Fig. 4a. Results showed that the free enzyme rapidly lost its activity, while the immobilized protease showed more stability during this experiment. Results showed that the protease@MOF reserved about 70 % of its original activity after 24 days of incubation, while the free enzyme only showed 22 % of its initial activity. Furthermore, the protease@MOF showed 2.11 folds more stability than the free enzyme after 14 days of incubation. Wang et al. [2020] reported that the activity of free protease decreased severely and retained 63.96 % and 33.91 % of the original activity after 12 and 27 days, respectively [56].

The reuse efficiency of immobilized protease@MOF was measured for 10 cycles. Results in the 10th cycle showed that the immobilized protease@MOF retained about 70 % of initial protein loading (Fig. 4b). Protein determination by the Bradford method in the 10th cycle exhibited that the immobilized CLEAs-Fib enzyme displayed 22 % leaching in the supernatant (Fig. 4b). This high operative enzyme

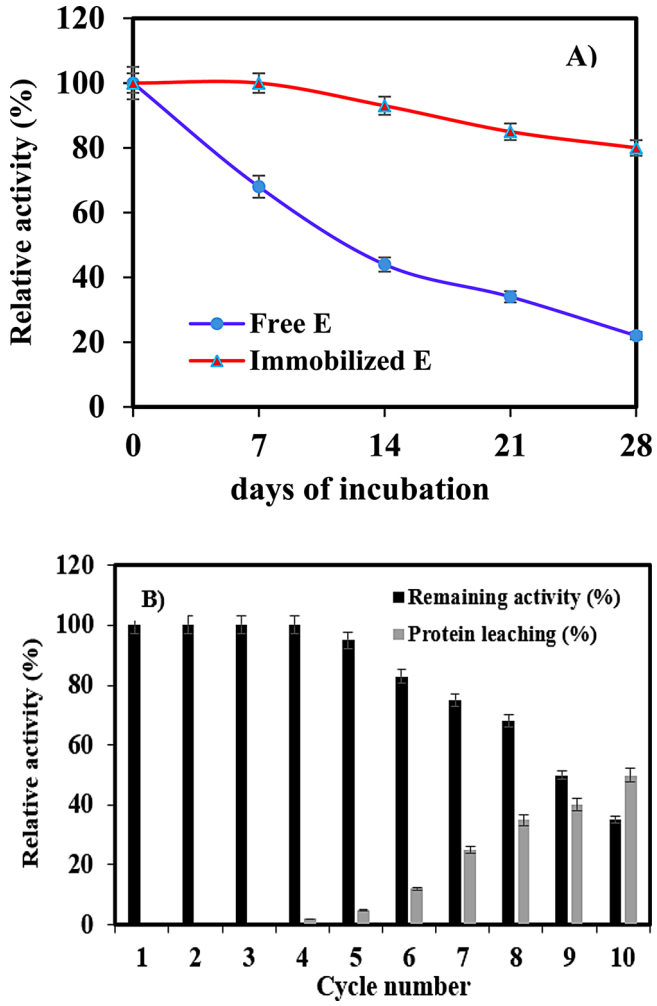

Fig. 4. a) Storage stability of free and immobilized proteases in different days of incubation, b) protease activity and leaching of the immobilized enzyme in subsequent reusing.

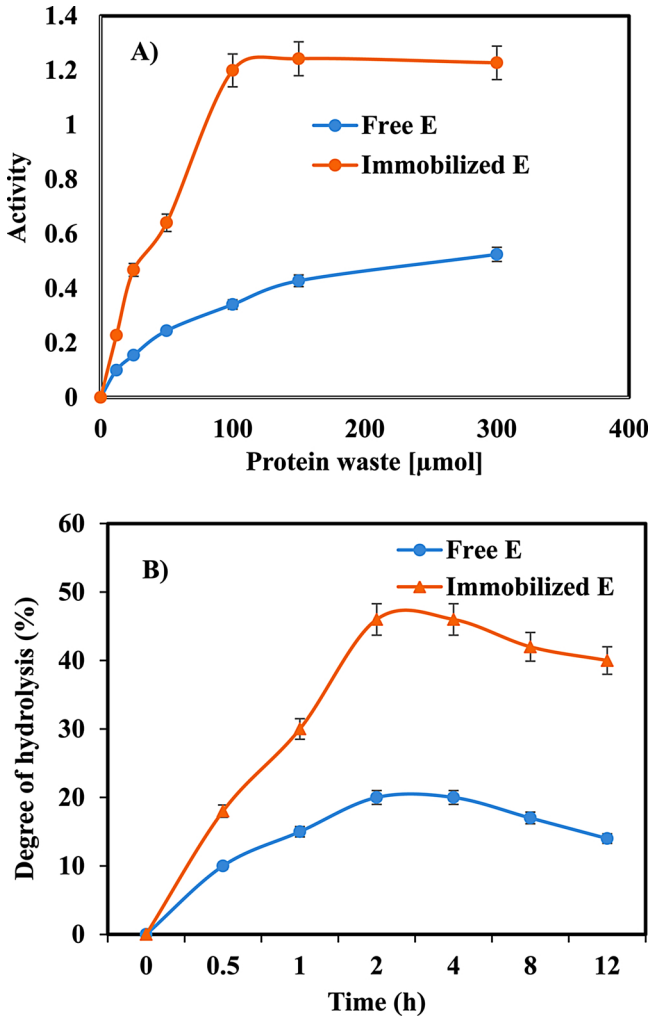

Fig. 5. a) protease activity of free and immobilized proteases in the presence of the different concentrations of protein fish-waste, b) Time course of hydrolysis degree of free and immobilized proteases in the presence of protein fish-waste.

stability could considerably decrease the cost of industrial synthesis.

These results proposed that the firmly captured enzyme in MOFs structures reserved the enzyme in the active conformation, and escaped the enzyme denaturation [77]. Furthermore, the reduction in the protease activity of enzyme@MOF was because of the development of several covalent connections between enzyme molecules [56].

3.6. Protease hydrolysis of protein fish-waste

Protease hydrolysis of protein isolated from *Thunnus tonggol* waste by free/immobilized proteases is shown in Fig. 5. Results showed that the immobilized enzyme displayed 2.9 folds more activity that the free enzyme in the presence of 150 µmol protein waste (Fig. 5a). Time-dependent protein hydrolysis by free and immobilized proteases showed that whole protein was decreased over time increasing. The hydrolysis degree of immobilized and free enzymes was gained about 46 and 20 % after 2 h of incubation at 50 °C, respectively (Fig. 5b).

In our previous report, we found that DH for Whey protein waste was 25 and 44 % for free and immobilized proteases after 20 min of incubation at 60 °C, respectively [50]. Furthermore, the immobilized halophilic protease from *Bacillus* sp. EMB9 showed 35 % of DH after 30 min at 50 °C [78]. Jemil and co-workers [2017] used *B. subtilis* A26 crude fibrinolytic protease to hydrolyze muscle proteins from sardinelle [9]. Their results showed that the hydrolysis reaction showed a high rate through the first 1 h, and then it was successively diminished, and then extended to a constant phase. Finally, it reached 10 % DH after 6 h. Previous studies indicated that the protein hydrolysates of fish waste showed some valued properties, including antihypertensive, antimicrobial, and antioxidant [4,8,79,80].

4. Conclusion

The free and immobilized proteases exhibited brilliant tolerance in the extensive range of pH and temperature. Also it had high stability in some surfactants, and oxidizing agents, whereas it belonged to the serine protease family. Results indicated that the higher stability and activity of the immobilized enzyme in different conditions. The possibility of using protease CHA410 in the hydrolysis of fish protein waste was investigated. The hydrolysis degree of immobilized protease CHA410 in the hydrolysis of fish protein waste was obtained about 46 % after 2 h of incubation at 50 °C. In comparison, it was gained about 20 % for protease CHA410 in the same condition. Taken together, these results indicated the high capacity of free and immobilized proteases in the production of protein hydrolysate from protein waste.

CRediT authorship contribution statement

Arastoo Badoei-dalfard: Supervision, Conceptualization, Methodology, Validation, Writing - review & editing. **Shima Khankari:** Data curation, Methodology, Validation. **Zahra Karami:** Methodology,

Validation, Writing - review & editing.

Declaration of Competing Interest

There is no declaration of interests.

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