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Immobilization of IMP-1 metallo-beta-lactamase: the promising approach for the removal of beta-lactam antibiotics

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ABSTRACT

Metallo-beta-lactamase enzymes are produced by some bacteria which can hydrolyze beta-lactam antibiotics such as penicillins, cephalosporins, cephamycins, and carbapenems. As a result, these enzymes cause bacteria resistance to these antibiotics. In this study, for the first time, IMP-1 metallo-beta-lactamases enzyme was immobilized on CNBr-activated sepharose through covalent bonding. The optimum temperatures of enzyme activity for free and immobilized enzymes were 70°C and 60°C. The optimum pH of IMP-1 activity was 7.5 that after immobilization, changed to 6.5. Due to enzyme immobilization, K_m and V_{max} values were decreased from 727.9 to 230.5 μmol and from 71.47 to 10.76 μmol/min for penicillin G as substrate. After 20 reaction cycles, 75% of the enzyme activity was retained, according to a reusability analysis of the immobilized enzyme. Furthermore, as compared to free enzyme, the thermal and storage stability of immobilized enzyme was reduced. Consequently, immobilization of IMP-1 by covalent binding on carriers suitable as a novel biocatalyst can be applied as a promising approach with high efficiency and inexpensive for the removal of antibiotics in hospital and pharmaceutical wastewater.

Keywords: Metallo beta-lactamase; Enzyme immobilization; Antibiotic pollution; Reusability; Biocatalyst

1. Introduction

Concerns about the presence of pharmaceutical compounds as part of emerging pollutants have been growing in the last three decades [1]. Various types of residues of medicinal compounds are found in ambient waters, including wastewater, surface water, and drinking water, at relatively low and consistent concentrations [2]. Antibiotics are one of the most important environmental pollutants, and because of their unique physical and chemical structure, they are called priority pollutants [3]. One of the harmful effects of these compounds is the development of microbial resistance to antimicrobial drugs. Tens of thousands of deaths from antibiotic-resistant bacteria occur

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each year, and the trend is increasing [4]. Beta-lactam antibiotics include cephalosporins, penicillins, carbapenems, and monobactams, which have a ring called beta-lactam in their molecular structure. This group is one of the most commonly used antibiotics in the world, which their residues can enter the water resources through urban and industrial wastewaters [5]. For this reason, it is essential to remove these compounds from wastewater to avoid environmental and public health risks [6]. Various methods were studied to remove drug compounds. One of the most promising methods to existing treatment technologies for removing these recalcitrant compounds is the application of specific enzymes [1].

Enzymes are biocompatible and biodegradable protein biocatalysts from renewable sources which operate under mild conditions at room temperature, atmospheric pressure, and physiological pH of high selectivity in the water solutions. As a result, they are sustainable, environmentally friendly, and cost-effective [7]. Enzymes may be used to clean up the environment in a variety of ways. First, enzyme technology is a "cleaner" option, since it replaces several industrial chemical processes that are harmful to the environment. Second, enzymes are directly utilized in waste management to improve the efficiency of wastewater treatment facilities. Third, they're employed as research aids [8]. Thus, enzymes may be unstable and difficult to control when exposed to abnormal conditions, and their selective function toward synthetic substrates may be poor. For most industrial uses, enzymes must be immobilized through simple and cost-effective instructions and used for reusing over long periods. Enzyme immobilization also usually results in catalyst stability against chemical and temperature changes [8,9].

Various materials can be used as a support for enzyme immobilization. These materials are generally classified into organic, inorganic, and hybrid or composite [1,10]. Sepharose is a trading name for agarose beads. Agarose is a polysaccharide produced from seaweed. To immobilization of ligands containing primary amines, provides a very convenient way to immobilize the ligands by the cyanogen bromide method. The coupling reaction is spontaneous, easy, and fast. The low cost, and good stability of agarose over a wide pH range, as well as the low non-specific binding of this material for many biological agents make agarose attractive as a support for many affinity chromatography applications. Sepharose-activated form of cyanogen bromide has many applications such as chromatography separation of biomolecules, immobilization of proteins, peptides, and nucleic acids [11,12].

Some bacteria can produce beta-lactamase enzymes that can hydrolyze β -lactam antibiotics. But, the carbapenems are somewhat resistant to beta-lactamases. These enzymes open the beta-lactam ring by hydrolysis; therefore, it neutralizes the antibiotic properties of these drugs. Beta-lactamases are classified into four classes. The three classes A, C, and D, are called serine beta-lactamases (SBLs) because they have a nucleophilic serine amino acid in their active site responsible for hydrolyzing the β-lactam ring [13]. Class B metallo-beta-lactamases have three subclasses: B1, B2, and B3 according to the overall geometry and homogeneity of the active site [14]. These enzymes need one or two zinc ions for activity. IMP-1, subclass B1, is an enzyme encoded by the transmissible *blaIMP* gene from *Pseudomonas aeruginosa*, which has the binuclear form (two zinc ions in the active site) [15].

In most previous studies, the effect of inhibitors of beta-lactamase enzymes to reduce antibiotic resistance has been investigated [16,17]. Only a few studies were performed using these enzymes to remove the antibiotics from water solutions [18,19]. The purified IMP-1 was immobilized for the first time. This study aims to develop of novel biocatalyst via the immobilization of IMP-1 metallo beta-lactamase on the CNBr-activated sepharose as a model system for IMP-1 immobilization by covalent bonding, and the feasibility of its use to remove beta-lactam antibiotics from aqueous solutions. Penicillin G was used as a representative of this group of antibiotics. Parameters kinetics (K_m, V_{max}), reusability, thermal and storage stability, and optimum temperature and pH were determined for this enzyme.

2. Materials and methods

2.1. Chemical material

CNBr-activated Sepharose was purchased from Sigma-Aldrich (USA), penicillin G (Pen G) was obtained from Bio Basic (Canada). Pen G is abbreviation of penicillin G. Other chemicals with laboratory-grade were purchased from Merck (Germany).

2.2. Enzyme expression and purification

Recombinant IMP-1 enzyme was expressed and purified based on the protocol Arjomandi et al. [20]. In brief, a pET28a plasmid harboring the bla_{IMP-1} gene from *Pseudomonas aeruginosa* was used for the transfer to competent BL21(DE3) *Escherichia coli* cells in the LB (Luria Bertani) broth culture medium by the heat-shock method. The cells were cultured at 37°C in LB broth enriched with kanamycin (50 μg/mL) as selecting an agent with shaking until the optical density (OD) 600 nm reached 0.4–0.6. Then, protein expression was induced by adding IPTG (Isopropyl β-D-1-thiogalactopyranoside) with the final concentration of 0.1 mM for 8 h at 25°C incubation. The culture was for further incubated for 2 h, and centrifuged at 5,000 × g for 10 min, and the cells were placed at –20°C for 24 h. Afterward, the cells were suspended in lysis buffer (pH 7.5) containing 50 mM Tris, 300 mM $NaCl_2$, and 5 mM imidazole. Protease inhibitor without EDTA, phenylmethylsulfonyl fluoride (PMSF), was added every 30 min for three times. Hence, the cells were disrupted by a sonicator (BANDELIN SONOPULS HD 2200, Germany). The lysate was clarified by centrifugation at 12,000 for 30 min to eliminate the cell debris. The protein solution was loaded onto Ni-NTA column. Afterward, the protein solution was dialyzed against a dialysis buffer containing potassium phosphate 50 mM, NaCl 300 mM, and $ZnCl_2$ 100 μM for 72 h at 4°C.

2.3. Enzyme and protein assay

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) is used to evaluate the enzyme's purity). For the IMP-1 activity test, an endpoint technique was applied. Purified enzyme, Pen G as substrate, and assay buffer (pH 7.5, 50 mM potassium phosphate buffer, and 100 mM $ZnCl_2$) were used in a final volume of 1 mL at 25°C. After 10 min

incubation, 100 μL EDTA (1 mM) was added to inhibit enzyme activity with chelating zinc ions. Enzymatic activity was evaluated by reducing the adsorption of Pen G resulting from the opening of the b-lactam ring at 235 nm wavelengths by using a Perkin Elmer Lambda 35 UV-Visible Spectrometer (USA). The results are presented as the average three standard deviations based on three independent measurements. A standard curve was prepared with various concentrations of Pen G in an assay buffer. The amount of enzyme which can cause hydrolysis 1 μmol Pen G per minute is determined as one unit of the enzyme activity. Bradford method with bovine 121 serum albumin (BSA) as standard was used to determine protein concentration [21].

2.4. Enzyme immobilization

IMP-1 immobilization on CNBr-activated Sepharose was carried out according to the study of Karami et al. 2020 [22]. In brief: 4.74 mg purified IMP-1 enzyme was added to a 1.5 mL 0.1 M $NaHCO_3$ buffer containing 0.5 M NaCl pH 8.3–8.5 as a coupling buffer. 0.3 g of CNBr-activated Sepharose resin was washed and swollen with cold 1 mM HCl. In general, 200 mL of 1 mM HCl per gram of dry weight of the resin was added. The supernatant was separated by gentle filtration (0.42-micron glass microfiber). Then, the resin was washed several times with distilled water, rinsed with 1.5 mL $NaHCO_3$/NaCl as coupling buffer, and instantly transferred to the ligand solution in the coupling buffer. The protein was mixed with the gel for 2 h at ambient temperature using a paddle stirrer and washed again with sodium $NaHCO_3$/NaCl to separate the unbound proteins. Non-reacted groups were blocked by glycine (0.2 M pH 8.0) for 2 h at ambient temperature. The blocking solution was removed by washing with a basic coupling buffer (pH 8.5), then with acetate buffer (0.1 M, pH 4) containing 0.5 M NaCl. These wash stages were completed several times with high and low pH buffer solutions. Then 200 μL of $ZnCl_2$ solution 500 μmol per mL of gel was added to supply Zn ions to the enzyme. Immobilization yield % (the ratio of covalently attached enzyme in carrier/enzyme used × 100) and activity recovery % (the ratio of the immobilized enzyme activity to free enzyme activity) of immobilized enzyme were calculated as follows:

$$\text{Activity recovery}(\%) = \frac{\text{immobilized enzyme activity}}{\text{free enzyme activity}} \times 100 \quad (1)$$

$$\text{Immobilization yield}(\%) = \frac{P_0 - P}{P_0} \times 100 \quad (2)$$

where P is protein concentration in the supernatant after the immobilization and P_0 is protein concentration in immobilization solution.

2.5. Characterization of free and immobilized IMP-1

2.5.1. Optimum temperature of enzyme activity and thermal stability

To determine the optimum temperature of immobilized and free enzymes activity, a certain amount of each enzyme was incubated in the assay buffer in the presence of 1 mM Pen G as a substrate in 1 mL of final reaction volume at 20°C–80°C for 15 min. After the end of each reaction time, 100 μL EDTA (1 mM) was added to inhibit free enzyme activity, and the immobilized enzyme was also separated from the reaction solution by centrifugation. The residual concentration of Pen G was determined by spectrophotometry according to the method mentioned in part 2–3, and the enzyme activity was calculated.

The thermal stability of free and immobilized IMP-1 was determined as follows:

Immobilized and free enzymes were incubated at 50°C, 60°C, and 70°C for different periods (0–60 min) in the absence of Pen G. Then, activity was measured at assay buffer as described earlier. The enzyme activity before incubation time was taken as the control (100% active).

2.5.2. Optimum pH of enzyme activity

Optimum pH for Free and immobilized enzyme activity was determined at 25°C for 15 min by 50 mM citrate (pH 5.5–6), 50 mM phosphate (pH 6.5–8), and 50 mM carbonate-bicarbonate buffers (pH 9–10). Residual activity was measured as described earlier.

2.5.3. Kinetic parameters

To obtain the values of K_m and V_{max} kinetic parameters, IMP-1 activity was measured at concentrations of 200–800 μM Pen G in assay buffer at 25°C for 5 min. Residual activity was measured as described earlier. These parameters were determined using Lineweaver-Burk and Michaelis-Menten plots by Prism-GraphPad6 software.

2.5.4. Reusability of the immobilized enzyme

A specified quantity of enzyme carrier sepharose was utilized for the enzymatic reaction according to the previously indicated approach to test the reusability of the immobilized IMP-1. At the end of each reaction, the immobilized IMP-1 was recovered by centrifugation at 1,000 × g rpm for 5 min, washed several times with assay buffer, and dispersed in the selfsame buffer as fresh. Then, the enzyme activity was determined, and the enzyme activity in the first cycle was considered 100%.

2.5.5. Storage stability of the immobilized enzyme

The storage stability of free and immobilized enzymes was determined in assay buffer at 4°C for 4 weeks. IMP-1 residual activity was determined as previously described at intervals of once a week. The activity of immobilized and free enzymes was calculated as a percentage of the initial activity in the $\text{time}_{(0)}$.

2.6. CNBr-activated Sepharose surface morphology

CNBr-activated Sepharose beads surface morphology was determined before and after the stabilization process using SEM analysis.

2.7. Statistical analysis

$P \leq 0.05$ was considered statistically significant. All experiments were performed independently with three replications to calculate the mean and standard deviation. All data related to immobility are expressed as means ± standard deviation. SPSS version 16 was used for statistical analysis.

3. Results and discussion

3.1. Enzyme expression and purification

To obtain and use of enzyme for the purposes of present study, the bla_{IMP} gene was cloned into a T7 expression vector (pE28a) and was overexpressed in the Escherichia coli BL21 (DE3). SDS-PAGE data showed that IMP-1 was expressed successfully. The molecular weight of the IMP-1 was approximately 29 kDa as determined by SDS-PAGE. The amount of purified enzyme obtained was 34 mg/L of culture medium, which is almost similar to the amount reported in the study of Laraki et al., 1999, 35 mg/L culture [23].

3.2. Enzyme immobilization

The enzyme metallo betalactamase IMP-1 was immobilized as a novel biocatalyst which is capable of hydrolyzing beta-lactam antibiotics from aqueous solutions. For this purpose, CNBr-activated Sepharose was used as a model system to immobilize the enzyme IMP-1. In this method, due to the reaction of cyanogen bromide with hydroxyl groups on agarose (sepharose), imidocarbonate esters or cyanate are formed. These groups can react with the primary amines under moderate conditions and leads to covalently ligand binding on the agarose matrix [24]. IMP-1 enzyme immobilization data are shown in Table 1. An amount of 4.74 mg of the purified enzyme was immobilized in 1 mL of Sepharose gel. At this concentration, the immobilization yield and activity recovery percentages were 100 and 15.156, respectively. Immobilization yield indicates that Sepharose gel is not yet saturated at this concentration of enzyme. With increasing the initial concentration of protein (enzyme), the quantity of free enzyme in the supernatant rises, and the percentage of immobilization yield falls, according to Eq. (2). Decreased activity of the immobilized enzyme can be due to external penetration restrictions, the transfer of the substrate from the solution bulk to the enzymes immobilized through the water boundary layer. Internal constraints are because the substrate must pass through a porous layer. These problems are reduced with increasing particle size, increasing the substrate concentration, and decreasing the boundary layer depth [25].

3.3. Characterization of the free and immobilized IMP-1

3.3.1. Optimum temperature of enzyme activity and thermal stability

The optimum temperature of the immobilized IMP-1 activity was 60°C, while for the free IMP-1, it was 70°C as indicated by Fig. 1A. However, no significant difference was observed in the immobilized enzyme activity at temperatures of 50°C, 60°C, and 70°C. The results showed that the thermal stability is decreased in the immobilized enzyme. The immobilized enzyme retains 75% of its activity after 1 h at 70°C, while this amount is about 83% for the free enzyme as indicated by the Fig. 1C and D. Decreased thermal stability of the enzyme after immobilization may be due to a variety of factors, including high loading of the support [26] and a change in the position of the enzyme on the porous surface of the support [27]. Surface immobilization may disrupt the structure of the enzyme due to interactions between the enzyme and the support, leading to reduced enzyme efficiency and stability [28]. The decrease in the optimum temperature activity of the immobilized IMP-1 can be attributed to the various factors that may cause these changes, such as changes in the three-dimensional structure of the enzyme that may occur during the immobilization process [29]. Despite the fact that the optimum temperature for immobilized enzyme activity was lower than for free enzyme, the data suggest that the immobilized form of IMP-1 may be employed for environmental applications that do not need high temperatures.

3.3.2. Optimum pH of enzyme activity

The optimum pH of the IMP-1 activity was 7.5 and shifted to 6.5 after immobilization, as indicated by Fig. 1B. These results correspond to a similar study, in which the optimal pH activity of TEM-1 beta-lactamase enzyme decreased from 7 to 6 after immobilization on the magnetic nanoparticles [18]. The immobilized enzyme in the optimum pH of the free enzyme activity was 93% active. The pH is one of the major parameters that can change enzyme activities in a reaction mixture. Immobilization usually affects the change of optimum pH due to conformational conversion in enzymes. This change in optimum pH of enzyme activity could have resulted from the alteration in basic and

Table 1
IMP-1 enzyme immobilization data

IMP-1 enzyme	Activity (U/mL)	Protein (mg/mL)
Free enzyme in immobilization solution	190.87 ± 3.872	2.56 ± 0.0081
Immobilized enzyme	28.847 ± 3.47	4.74 ± 0.0124
Enzyme in the supernatant after immobilization	0	0
	Activity recovery (%)	Immobilized yield (%)
	15.156 ± 2.122	100

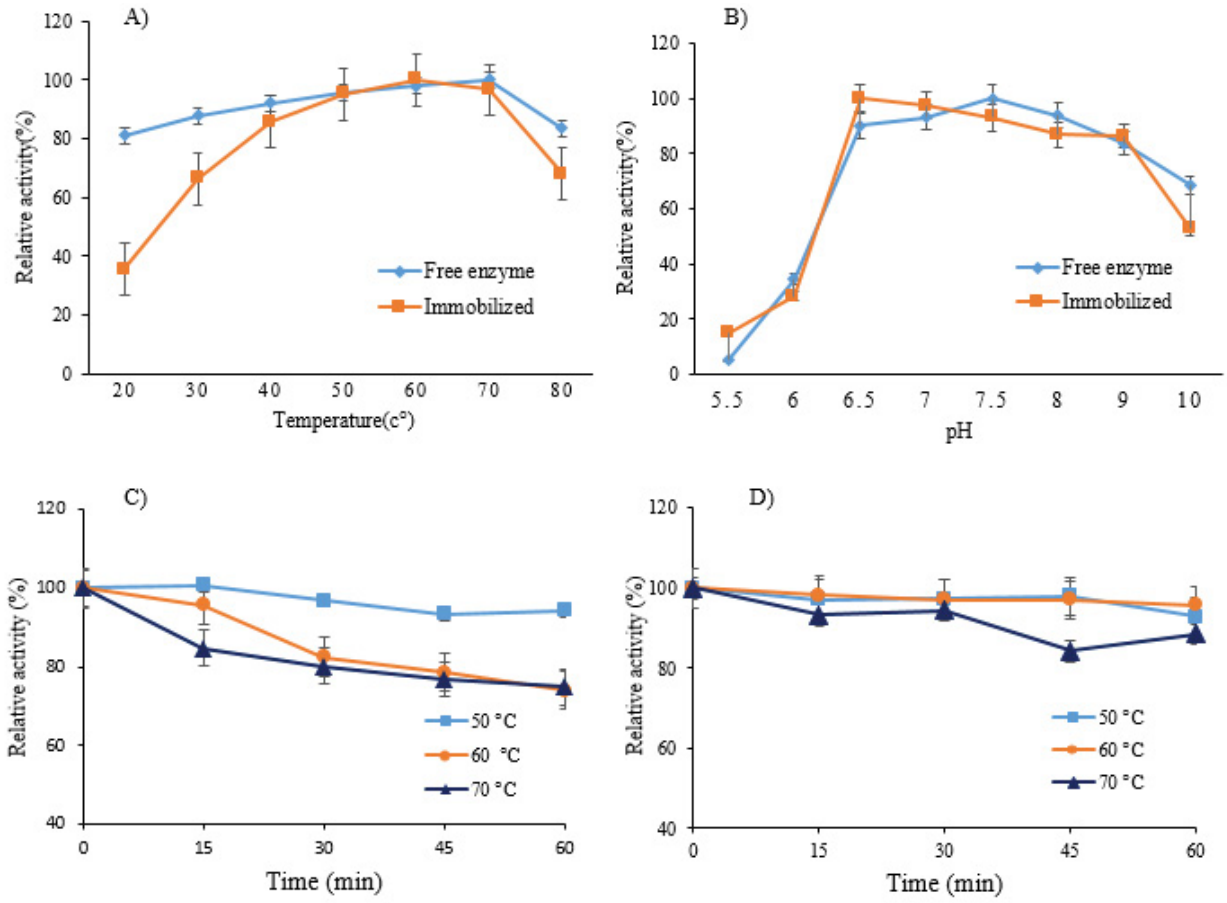

Fig. 1. (A) The optimum temperature of free and immobilized IMP-1 activity and (B) optimum pH of free and immobilized IMP-1 activity, (C) thermal stability of immobilized IMP-1, and (D) thermal stability of free IMP-1.

acidic amino acid side chain ionization in the surroundings around the active site [30].

3.3.3. Kinetic parameters (K_m and V_{max}) of the free and immobilized enzymes

Michaelis constant (K_m) is the concentration of the substrate when the reaction velocity is half the maximum velocity. In enzyme kinetics, V_{max} is the maximum velocity of an enzymatically catalyzed reaction when the active sites of enzyme are saturated with its substrate. The K_m and V_{max} values of immobilized and free IMP-1 were studied by Michaelis–Menten, and Lineweaver-Burk plots. These plots are shown in Fig. 2. K_m and V_{max} values for free IMP-1 were 728 and 71.75 μmol respectively, and 230.5 and 10.76 μmol/min for the immobilized, respectively. The decrease in the value of V_{max} may be due to the substrate diffusion limit [31]. This manner has been reported in other studies for enzymes that are immobilized in various carriers [24,32]. Moreover, after immobilization of IMP-1 on the carrier, the K_m value significantly decreased which indicates an increase in the affinity of the enzyme for the substrate. The substrate affinity for the enzyme may be increased due to structural changes in the active site of the enzyme. However, another study on lipase immobilization on nanocomposite membranes showed a decrease in the K_m value [34].

3.3.4. Reusability and storage stability of the immobilized enzyme

The immobilization of enzymes has provided several advantages compared to free enzymes: stable biocatalysts, production of efficiency, the possibility of reuse, and easy purification of the products. One of the most important goals and of enzyme immobilization is the reusability of the immobilized enzyme. Immobilized enzymes can be recovered from the reaction mixture and made available again for reuse (cost-effectiveness). Immobilized IMP-1 maintained more than 75% of its initial activity after 20 cycles of reusing (Fig. 3A). In a similar study, the immobilized TEM-1 enzyme on the surface of silica-modified magnetic nanoparticles retained 95% of its initial activity after 35 cycles of reuse in the removal of penicillin G. This appropriate reusability can be the result of the type of carrier and the method of covalent binding the enzyme to the carrier through the spacer arm [18].

Free enzymes are generally less stable than immobilized enzymes, and their activities gradually decrease with time. The results of the experiments in this study showed that over time, the activity of both free and immobilized enzymes decreased. Immobilized IMP-1 retained more than 61% of its initial activity after 4 weeks of storage in 50 mM phosphate potassium buffer (pH 7.5) at 4°C, while the free enzyme retained about 77% of its initial activity after 4 weeks

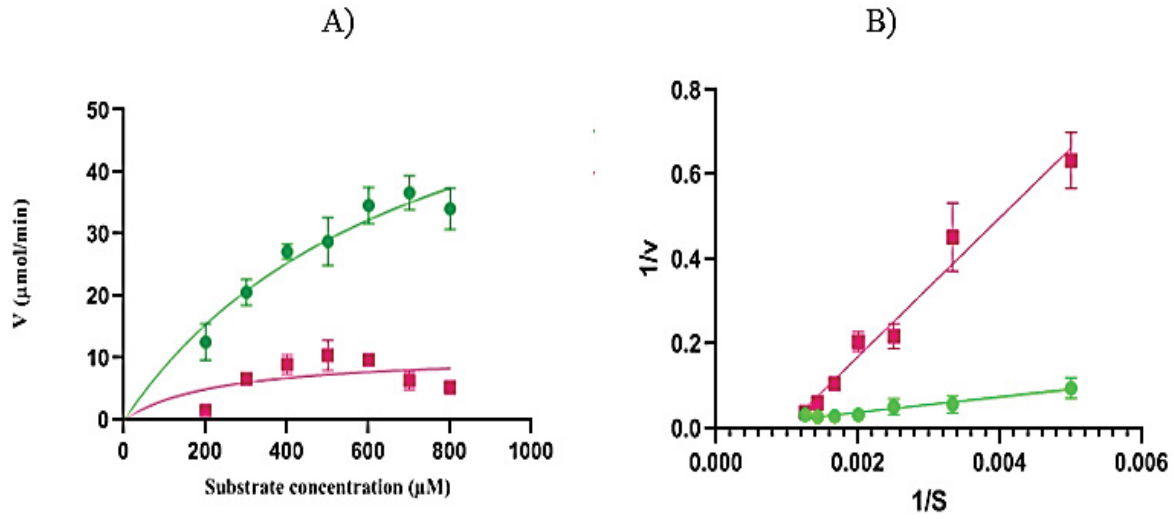

Fig. 2. (A) Michaelis–Menten plots of the free and immobilized IMP-1 and (B) Lineweaver–Burk plots of the free and immobilized IMP-1.

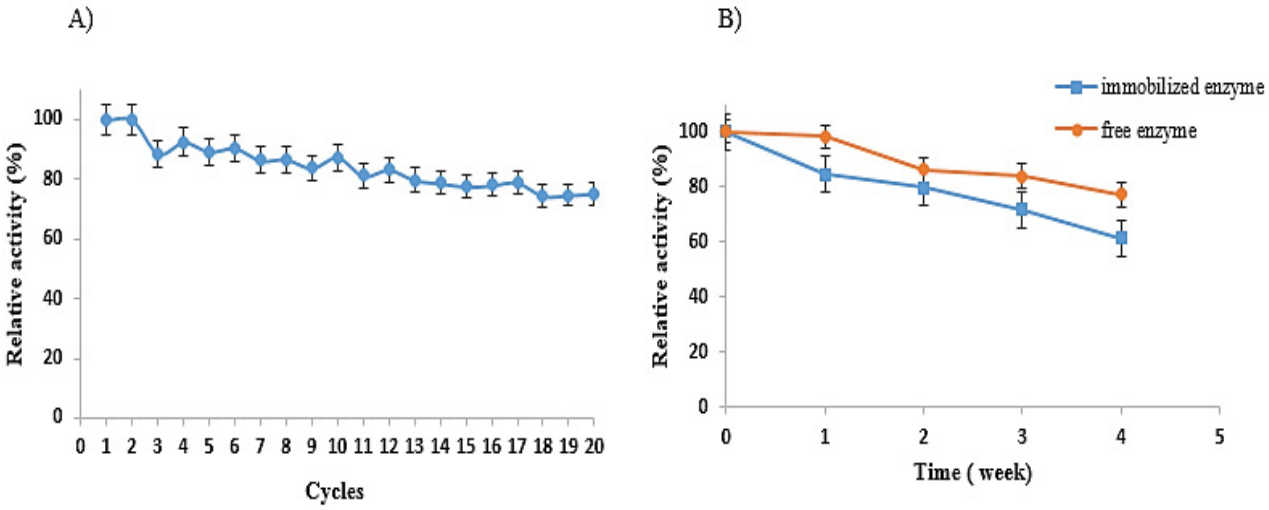

Fig. 3. (A) Recyclability of immobilized IMP-1 and (B) Storage stability of the free and the immobilized IMP-1.

as indicated in Fig. 3B. This decrease in storage stability of immobilized IMP-1 to the free enzyme may have been in terms of a change in the structure of the Sepharose as an enzyme carrier due to storage time which has led to enzyme leakage from the carrier. Therefore, it can be concluded that Sepharose as an enzyme carrier is not very suitable for long-term procedures [33].

3.4. CNBr-activated sepharose surface morphology

It is important to maintain the original structure and morphology of the enzyme support during the various stages of enzyme immobilization. The results of SEM analysis showed that the morphology of CNBr-activated Sepharose was preserved before and after enzyme immobilization, as indicated by Fig. 4).

4. Conclusion

The enzyme IMP-1 was successfully immobilized for the first time. Cyanogen bromide-activated Sepharose was used as a system model to immobilize this enzyme by covalent bonding, which decreased thermal stability, storage stability compared to free enzyme. Enzyme immobilization can be a way of saving costs by improving reusability, which is the most important purpose of enzyme immobilization. In addition, the recovery and separation of the immobilized enzymes are convenient.

The kinetic parameters of the free and immobilized enzymes showed a significant decrease in K_m and V_{max}. Furthermore, the optimum pH of the enzyme changed to slightly acidic after immobilization. Activity recovery of enzymes decreased after immobilization. These enzymes, specifically hydrolyze only the beta-lactam ring of the

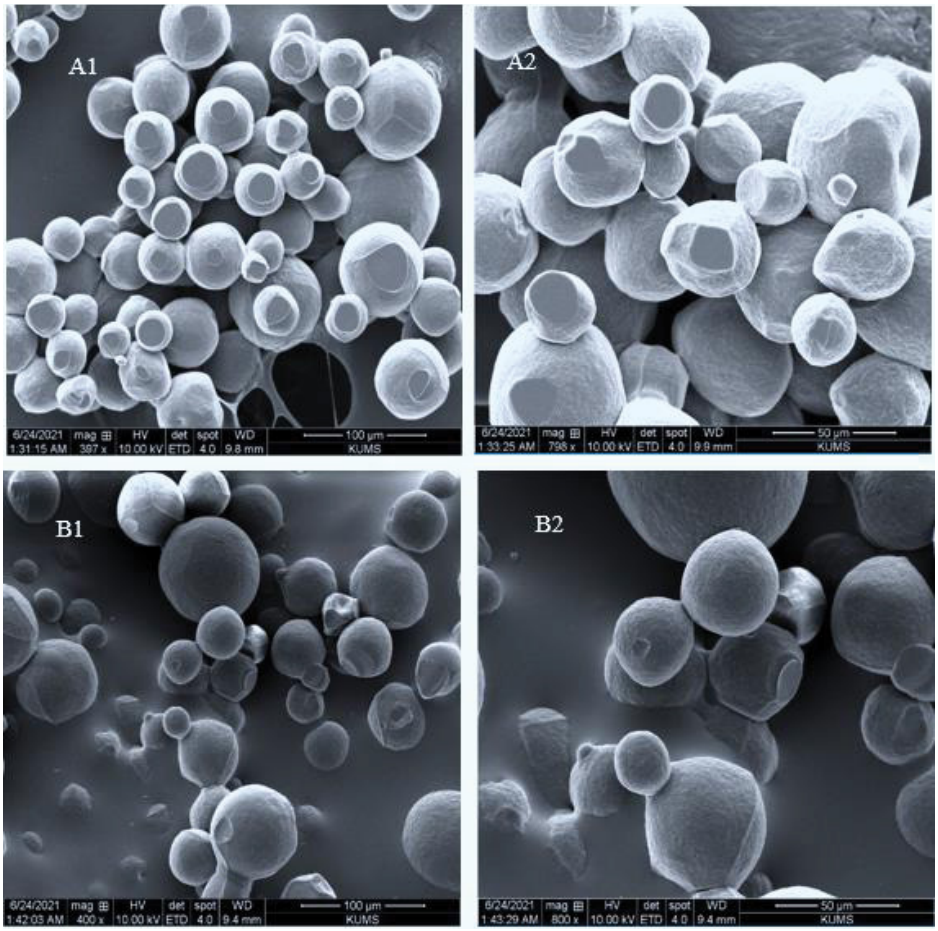

Fig. 4. Representative SEM images of sepharose beads at different magnifications: before immobilization (A1 and A2), after immobilization (B1 and B2).

antibiotic and neutralize its antibacterial effect, and hazardous by-products are not produced. It can be concluded that IMP-1 enzyme immobilization can be improved by covalently binding to more suitable carriers and other immobilization methods, such as the use of a spacer arm between the enzyme and the carrier. Therefore, it can be concluded that immobilized IMP-1 can be used as a promising approach with high efficiency and inexpensive, through continuously operated in a batch processing by a convenient separation for treatment of antibiotics polluted-water.

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Conflict of interests

The authors declare that they have no conflict of interest regarding the publication of the current study.

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