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Degradation Pathway of Penicillin by Immobilized β-lactamase

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The ever-emerging environmental pollution as a result of the abuse of antibiotics and other pollutants has caused a serious threat to the ecological environment and human health. Therefore, development of effective strategies for degradation, as well as disposal of antibiotic residues in water is urgently needed. Antibiotics, such as penicillins, are extensively utilized for treatment of bacterial infections for humans and animals and have been spreading in water due to their extremely low metabolic rate. Immobilized β-lactamase onto aminosilane magnetic nanoparticles (AMN-MNPs) was used for the degradation of penicillin (PEN). Thermal stability of such immobilized β-lactamase was investigated, as well as enzyme kinetics of free β-lactamase and immobilized β-lactamase was determined. Degradation study of PEN was performed with free and immobilized β-lactamase and analyzed using HPLC system. The obtained results show that immobilized β-lactamase has many advantages compared to free β-lactamase. The immobilized enzyme exhibited hyperactivation of the enzyme, resulting in 107 % residual activity with 100 % immobilization efficiency. Immobilized β-lactamase showed good thermal stability at various temperatures above 40°C when compared to free β-lactamase, which was inactivated when exposed to temperatures above 40°C. The results also suggest that the catalytic activity of immobilized β-lactamase for the degradation of PEN has been significantly enhanced compared to free β-lactamase, resulting in 98 % degradation of PEN, while only 22 % of PEN was degraded with the free enzyme after 24 hours.

1. Introduction

Residues of antibiotics accumulating in different water resources are becoming an important environmental and public health issues. As these pollutants are discharged into the environment through various ways, the pharmaceutical industry is therefore one of the main sources of such releases of pollutants into the water sources (Manoharan et al. 2022). The reduction and elimination of such antibiotic wastes are important, due to the development of possible antibiotic resistance (Feng et al. 2021). Wastewater is a major source of antibiotics, and many methods have been investigated for their degradation and removal. ß-Lactam antibiotics are the most common drugs used in bacterial therapy since the discovery of penicillin. Despite the enormous quantity of antibiotics in pharmaceutical industry available nowadays, bacteria usually develop defense mechanisms (Nkoh et al. 2023). Methods for removing antibiotics from wastewater must have high efficiency and low disposal costs (Werkneh and Islam 2023). Nowadays, we are paying more and more attention to biological methods, such as the removal of antibiotics with the help of enzymes. However, native enzymes are poor for practical use due to their difficult reusability and instability under harsh conditions. Therefore, some strategies, such as enzyme immobilization have been developed to improve their catalytic performance and increase their stability and reusability (Singh and Bhardwaj 2024). Enzymes can catalyze and degrade antibiotics (Reisoglu and Aydin 2023) with the advantage of excellent catalytic features, high biocompatibility and eco-friendly performance (Yang et al. 2021; Soni et al. 2001).Penicillin (PEN) is effective against many bacterial infections and is one of the most used antibiotics in the world. Different types of PEN are known. The basic structure of each PEN consists of a β-lactam and a thiazolidine ring and a side chain. As with other types of antimicrobials, the widespread use of β-lactams has led to the development of defense mechanisms (Jampani et al. 2024; Nassri

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et al. 2023).The expression of ß-lactam degrading enzymes, β-lactamases, is the most common mechanism of antibiotic resistance among bacteria. Moreover, ß-lactam antibiotics are also toxic with only 20% degradability. ß-Lactamases are produced by bacteria that play an important role in their resistance to ß-lactam antibiotics due to their hydrolyzation of ß-lactam ring (D. Wang et al. 2021). Therefore, effective remediation of ß-lactam antibiotics is one of the most important requirements to prevent antibiotic pollution.

2. Materials and methods

2.1 Materials

The model antibiotic, Penicillin G potassium salt, enzyme β-lactamase from *Enterobacter cloacae*, glutaraldehyde ($OHC(CH_2)_3CHO$, 25 % m/v aqueous solution), AEAPS ($C_9H_{23}NO_3Si$, >99 %), methanol (CH_3OH, ACS grade), potassium phosphate (KH_2PO_4), sodium hydroxide (NaOH, 93 %), and hydrochloric acid (HCl, 37 %), bovine albumin serum (BSA), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 99.5 %) were supplied by Sigma Aldrich (Germany). Dimethyl sulfoxide (DMSO) and methanol (HPLC grade) were acquired from Merck (Germany). All other chemicals were of analytical grade and used as received without any further purification.

2.2 Synthesis of aminosilane magnetic nanoparticles (AMN-MNPs)

Synthesis of AMN-MNPs with silanization protocol of prepared AMN-MNPs was performed as described in our previously published research (Šulek et al. 2010). Briefly, the maghemite nanoparticles were prepared by co-precipitation reaction, containing a solution mixture of ferrous (Fe^{2+}) and ferric (Fe^{3+}) ions in ratio 1:2, which was prepared in the presence of a reducing agent, ammonia solution (NH_4OH) with constant stirring. For the experiments to prepare maghemite nanoparticles of uniform size, the initial pH of the iron salts mixture was adjusted to a particular value of 1.5 and kept constant for 30 min. Later, the pH was increased rapidly to 11 by adding 25 % of ammonia solution directly into solution of iron salts and was stirred for another 30 min with vigorous stirring. The colour of bulk turned from orange to black immediately. The maghemite nanoparticles, precipitated at the bottom of a stirred-tank batch reactor equipped with a mechanical stirrer were washed few times with miliQ water by magnetic decantation. Moreover, surfactant coating procedure was carried out at elevated temperature, 75 °C for 90 min. After surfactant coating, the maghemite nanoparticles were centrifuged at rotational speed of 3000 rpm for 5 min and the precipitates at the bottom of a centrifuge tube were removed, whereas the magnetic fluid composed of stable maghemite nanoparticles was utilized in the next step of surface functionalization with silica. In order to obtain a uniformly distributed functional layer of silica onto the surface of maghemite nanoparticles, 200 µL of sodium silicate was consecutively added into magnetic suspension. The reaction was allowed to proceed at 90 °C for 3 hours under continuous mechanical stirring. At the end, the suspension was cooled down and hydrochloric acid was added dropwise to adjust pH value to 7. Finally, the resulted AMN-MNPs were separated by a permanent magnet and dried at room temperature. Moreover, silanization reaction by amino silane coupling agent (AEAPS) took place in order to provide highly functionalized AMN-MNPs ready for covalent immobilization of β-lactamase.

2.3 Enzyme immobilization

10 mg of previously synthesized AMN-MNPs were functionalized with 100 µL glutaraldehyde (GA) in 900 µL of HEPES buffer. Functionalization was performed at 450 rpm for 1 hour. After functionalization, the supernatant was magnetically decanted using an external magnetic field. Functionalized AMN-MNPs were further immobilized with enzyme β-lactamase using concentration of 0.1 mg/mL. Immobilization was carried out on a rotary mixer at 450 rpm for 1 hour. Enyzme β-lactamase was stabilized with proteic feeder bovine serum albumin (BSA) at a concentration of 1 mg/mL in ration 1:3. 100 µL of such stabilized enzyme β-lactamase in HEPES buffer was immobilized onto functionalized AMN-MNPs. The immobilization was performed at 450 rpm for 2 hours. After immobilization, the supernatant was magnetically decanted and used for protein determination according to Bradford method.

2.4 Activity assay

β-Lactamase activity was assessed spectrophotometrically. The nitrocefin solution contained 5 mg of nitrocefin, 250 µL of DSMO, 4.75 mL of 25 mM HEPES buffer. The reaction that contained 570 µL of β-lactamase enzyme in 50 mM HEPES buffer was initiated with 30 µL of prepared nitrocefin. β-Lactamase activity was monitored by measuring the increase in absorbance at 482 nm for 1 min at room temperature.

2.5 Thermal stability of immobilized β-lactamase

An important characteristic of immobilized enzymes is thermal stability. The thermal stability of immobilized β-lactamase was determined after incubating immobilized β-lactamase at different temperatures. The immobilized

β-lactamase was incubated for 24 hours at temperatures of 40°C, 60°C and 80°C. After 24 hours of incubation, the activity of the enzyme was determined and compared with the activity determined for the immobilized enzyme before exposure. Thermal stability of the free enzyme was determined using the same procedure.

2.6 Assessment of PEN concentration

PEN degradation study was performed using 0.1 mg/mL of PEN solution with 20 mg of immobilized β-lactamase. Then, the mixture solution was incubated at room temperature for 120 min at 200 rpm. The remaining concentrations of PEN were monitored by HPLC method, using Agilent HPLC with an UltimateR XB C18 column (4.6 × 150 mm, 5 μm) at a flow rate of 1.0 mL min^{-1}, column temperature of 30 °C, mobile phase of methanol and phosphate (0.1 M potassium dihydrogen phosphate, pH 3.5) at a ratio of 50:50, injection volume of 20 μL and detection wavelength of 225 nm (P. Wang et al. 2021).

3. Results and Discussion

3.1 Thermal stability of immobilized β-lactamase

Immobilized β-lactamase resulted in hyperactivation, showing 107 % residual activity with 100 % immobilization efficiency when compared to free β-lactamase. Thermal stability of enzymes plays a key role in their usefulness in various industrial applications. The thermal stability of immobilized β-lactamase was compared with the thermal stability of free β-lactamase at temperatures of 40 °C, 60 °C and 80 °C. The results are shown in Table 1. The thermal stability of immobilized β-lactamase was determined according to its activity before exposure. After 24 hours of incubation at 40 °C, the activity of immobilized β-lactamase resulted in 54 %. As the exposure temperature increased, a decrease in the residual activity of the immobilized β-lactamase appeared. When incubation was carried out for 24 hours at 60 °C, it decreased to 47 %. A further decrease in the activity of the immobilized β-lactamase was observed when the temperature was increased to 80 °C, as the activity of the immobilized β-lactamase resulted in 28 %. When investigating the thermal stability of free β-lactamase, enzyme inactivation at all three temperatures after 24 hours of incubation occurred. From the obtained results, we can observe an increased thermal stability after immobilization of enzyme β-lactamase onto AMN-MNPs

Table 1: Residual activity of immobilized β-lactamase after 24 hours of incubation at 40, 60 and 80 °C.

Temperature [°C]	Residual activity [%]	
	Free β-lactamase	Immobilized β-lactamase
40	0 %	54 %
60	0 %	47 %
80	0 %	28 %

3.2 PEN degradation using immobilized β-lactamase

The catalytic performance of immobilized β-lactamase for degradation of PEN was estimated in water solution at room temperature. As shown in Figure 1, PEN degradation was determined with immobilized β-lactamase using enzyme concentration of 0.01 mg/mL. PEN concentration was 0.1 mg/mL, as well.

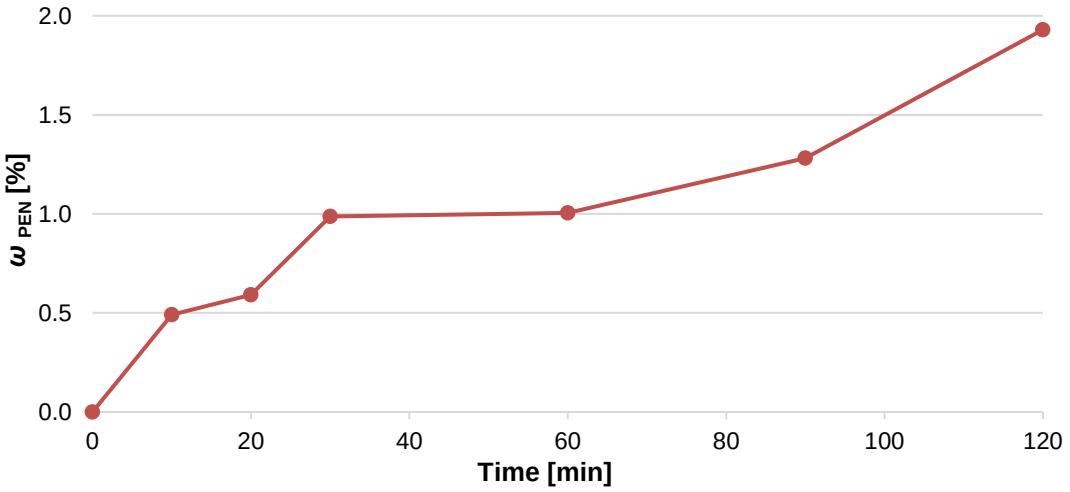

Figure 1: The amount of degraded PEN after 120 min by immobilized β-lactamase using 0.01 mg/mL of enzyme and 0.1 mg/mL PEN concentration.

From the results in Figure 1 we can observe that the degradation of PEN with immobilized β-lactamase was lower, as only 2 % of PEN was degraded after two hours, therefore the concentration of PEN in the solution reaction remained at 98 % after 2 hours. Since the degradation of PEN with immobilized β-lactamase at concentration of 0.01 mg/mL was very low, the concentration of immobilized β-lactamase was increased to 0.1 mg/mL, and furthermore the amount of AMN-MNPs was increased to 20 mg in the immobilization protocol, as well. Other immobilization conditions remained unchanged. From the results shown in Figure 2, it can be seen that the degradation of PEN with a 0.1 mg/mL concentration of immobilized enzyme was much higher. As the reaction time prolonged, the amount of degraded PEN increased. After 30 and 60 min, 7 % and 12 % PEN degradation was achieved, respectively, while after 2 hours only 26 % of PEN was degraded. After 5 hours 83 % of PEN degradation was observed. After 24 hours, almost complete PEN degradation was reached, resulting in 98 % degradation.

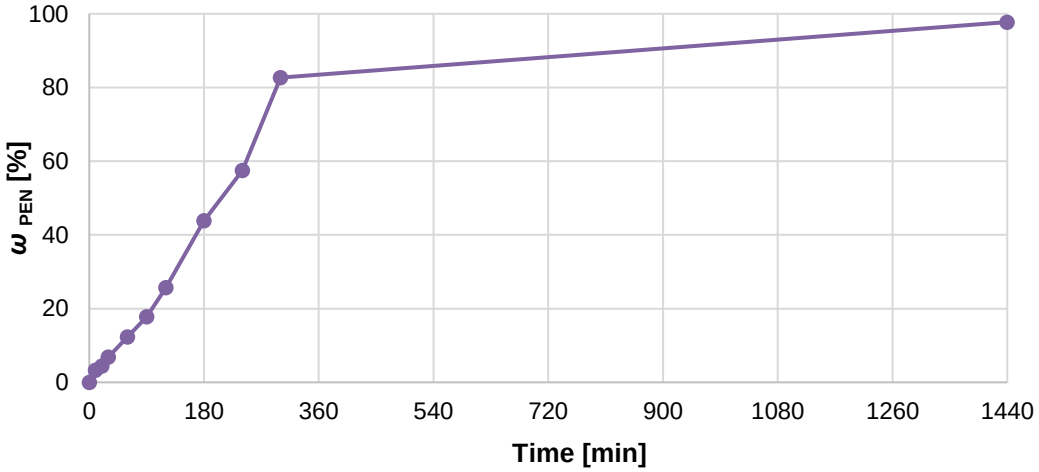

Figure 2: The amount of degraded PEN after 24 hours by immobilized β-lactamase using 0.1 mg/mL of enzyme and 0.1 mg /mL PEN concentration.

Figure 3 shows a comparison of the degradation rates of PEN (0.1 mg/mL) with free and immobilized β-lactamase and with bare AMN-MNPs, which served as a control experiment.

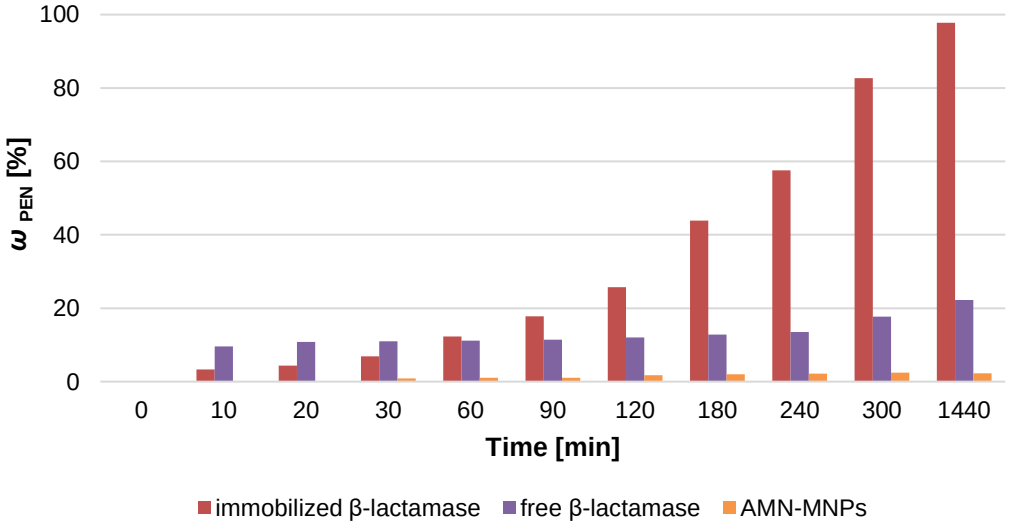

Figure 3: Comparison of percentages of degraded PEN after 24 hours at room temperature by free, immobilized β-lactamase and AMN-MNPs using 0.1 mg/mL of enzyme and 0.1 mg /mL PEN concentration.

The results show that the degradation of PEN (0.1 mg/mL) with free β-lactamase was lower than with immobilized β-lactamase at the same enzyme concentration. In the initial 30 min, the degradation of PEN was higher with free enzyme when compared to the immobilized one, as the degradation rate after 30 min with the free enzyme was 11 %, and with the immobilized enzyme only 7 %. However, we can see that PEN began to

degrade faster with additional time using the immobilized enzyme than with the free enzyme. After 3 hours, the degradation rate with immobilized β-lactamase was 44 %, while with free β-lactamase it was only 13 %. After 24 hours, we can see that the PEN degradation rate with the immobilized enzyme is 98 %, while only 22 % of PEN was degraded with the free enzyme after 24 hours. Most likely, due to the limitation in substance transfer, more time is needed at the beginning for the substrate to penetrate to the active sites on the enzyme, which is immobilized on the carrier. The control experiments suggest AMN-MNPs have only slight catalytic property for degradation of PEN (about 2.5 %) due to forming complexes between PEN molecule and AMN-MNPs functional groups. Results suggest that immobilized β-lactamase exhibits outstanding activity for degradation of PEN when compared to free β-lactamase. The degradation efficiency of PEN by immobilized β-lactamase increased significantly with increasing the reaction time, which suggests that it takes time for PEN to interact with covalently bound enzyme onto AMN-MNPs.

The effect of PEN concentration on degradation efficiency was also estimated under catalysis of immobilized β-lactamase in water solution. Figure 4 shows the remaining percentages of PEN with the time of degradation reaction for various concentrations of PEN, namely 0.5 mg/mL, 0.1 mg/mL and 0.05 mg/mL.

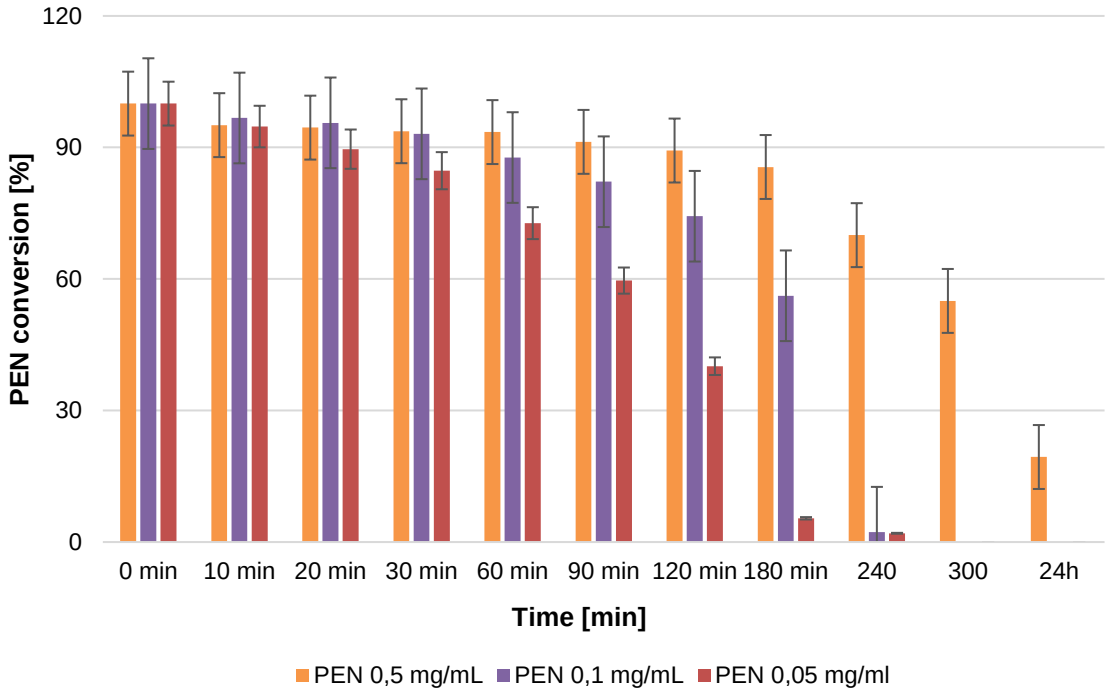

Figure 4: Comparison of PEN conversions with immobilized β-lactamase at different concentrations of PEN.

It can be observed that the degradation efficiency of PEN increased with prolonging the time of degradation reaction for all concentrations of PEN. Furthermore, the remaining percentage of PEN in the system gradually increased with decreasing the concentration of PEN. PEN degradation was the highest at PEN concentration of 0.05 mg/mL. At 0.05 mg/mL of PEN, the degradation after 30 min was 15 %, resulting in 85 % of remaining PEN in solution. At the same time the degradation of PEN with a concentration of 0.5 mg/mL was 6 %, resulting in 94 % of remaining PEN in reaction solution. When investigating the concentration of PEN at 0.1 mg/mL, only 7 % of PEN degraded. After 2 hours of degradation process, when using PEN concentration of 0.5 mg/mL, only 11 % of PEN degradation was achieved, resulting in 89 % of remaining PEN in reaction solution. However, when using PEN concentration of 0.1 mg/mL, 26 % of PEN degraded, remaining 74 % of PEN in reaction solution. After 3 hours, PEN degradation was already 95 % for PEN concentration of 0.05 mg/mL, while it remained at 15 % when using 0.5 mg/mL PEN concentration. Both PEN concentrations (0.05 and 0.1 mg/mL) efficiently degraded PEN after 4 hours, resulting in 96 % and 97 % of degradation, respectively. After 24 hours, all PEN was degraded, while when using PEN concentration of 0.5 mg/mL, 20 % of PEN remained in the reaction system. From the obtained results we can see that the higher the PEN concentration, the lower its degradation. The results are reasonable, since high concentrations of PEN antibiotic are beneficial to the catalytic reaction and suggest that immobilized β-lactamase has high catalytic activity for degradation of PEN even at lower concentrations.

4. Conclusions

Enzyme β-lactamase was immobilized onto AMN-MNPs and their thermal stability and catalytic performance with degradation pathway for PEN was investigated by HPLC method. The obtained results show that immobilized β-lactamase has many advantages compared to free β-lactamase. The immobilized enzyme exhibited good thermal stability at various temperatures above 40°C when compared to free β-lactamase, which was inactivated when exposed to temperatures above 40°C. The results also suggest that the catalytic activity of immobilized β-lactamase for the degradation of PEN has been significantly enhanced compared to free β-lactamase. This work provides a novel strategy for the biodegradation of the β-lactam antibiotics in the environment, as new approaches could lead to more efficient and cost-effective procedures for cleaning antibiotics from wastewater.

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