IJPST
Indonesian Journal of Pharmaceutical Science and Technology

Research Article
Vol. 12 No. 3 (2025): 272-279
https://doi.org/10.24198/ijpst.v12i3.55501

Immobilization of Penicillin-G Acylase from *Bacillus thuringiensis* BD1 for Enhanced Amoxicillin Production Using Na-Alginate Entrapment

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Abstract
Efficient enzymatic production, particularly using Penicillin-G Acylase (PGA), is crucial for synthesizing amoxicillin, a penicillin-type antibiotic. This study optimized PGA immobilization from *Bacillus thuringiensis* BD1 using Na-alginate to enhance stability and cost-effectiveness. Various Na-alginate concentrations (1%, 1.25%, 1.5%) were tested, with stability assessments at pH 6-9 and temperatures of 30-60°C, alongside reusability, morphology, and amoxicillin synthesis evaluations. Initial activity was 46.59 U/mg, with optimal immobilization at 1.5% Na-alginate achieving 41.01 U/mg. After four uses, immobilized PGA BD1 retained ±20% activity with optimal conditions at pH 7 and 40 °C. Enhanced amoxicillin synthesis compared to free enzymes highlights its industrial potential. This research demonstrates the feasibility of using immobilized PGA BD1 for scaling up amoxicillin production, offering significant economic and technological benefits.

Keywords: enzyme immobilization, entrapment, penicillin-G acylase, sodium alginate, amoxicillin

Article History:
Submitted 20 June 2024
Revised 06 March 2025
Accepted 12 March 2025
Published 31 October 2025

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Citation:
Dewi, R.A.P.; Widyasti, E.; Dianursanti, D; Sriherwanto, C.; Kusumaningrum, S.; et al. Immobilization of Penicillin-G Acylase from *Bacillus thuringiensis* BD1 for Enhanced Amoxicillin Production Using Na-Alginate Entrapment. Indonesian Journal of Pharmaceutical Science and Technology. 2025: 12 (3), 272-279.

Imobilisasi Penicillin-G Acylase dari *Bacillus thuringiensis* BD1 untuk Peningkatan Produksi Amoksisilin Menggunakan *Entrapment* Na-Alginat

Abstrak
Proses produksi enzimatik yang efisien sangat penting untuk sintesis amoksisilin, antibiotik tipe penisilin. Imobilisasi Penicillin-G Acylase (PGA) dari *Bacillus thuringiensis* BD1 menggunakan Na-alginat meningkatkan stabilitas dan efektivitas biaya, tetapi kondisi optimal masih perlu dieksplorasi. Penelitian ini mengoptimalkan imobilisasi PGA dengan Na-alginat, menilai stabilitas dan efektivitas dalam sintesis amoksisilin pada pH dan suhu tertentu. *Entrapment* Na-alginat diuji dengan konsentrasi 1%, 1.25%, dan 1.5%. Uji stabilitas meliputi pH 6-9 dan suhu 30-60 °C, serta evaluasi penggunaan ulang, morfologi, dan sintesis amoksisilin. Aktivitas awal sebesar 46.59 U/mg, dengan imobilisasi optimal pada 1.5% Na-alginat (41.01 U/mg). Setelah empat kali penggunaan, PGA BD1 mempertahankan ±20% aktivitas. Kondisi optimal pada pH 7 dan 40 °C. PGA BD1 yang diimobilisasi menunjukkan peningkatan sintesis amoksisilin dibandingkan enzim bebas, menunjukkan potensinya untuk aplikasi industri dan keuntungan ekonomi.

Kata Kunci: entrapment, imobilisasi enzim, penicillin-G acylase, sodium alginat, amoksisilin

1. Introduction

Amoxicillin, a widely used beta-lactam antibiotic, is an aminopenicillin designed to combat antibiotic resistance by adding an amino group to penicillin. It treats infections caused by beta-lactamase–negative bacteria, including respiratory, urinary tract, skin, and soft tissue infections, as well as *Helicobacter pylori* eradication. Amoxicillin is effective against a range of gram-positive and some gram-negative bacteria, including *Streptococcus* sp., *Listeria monocytogenes*, *Enterococcus* spp., *Haemophilus influenzae, Escherichia coli,* and others.[1]

Efficient enzymatic production processes are crucial for the synthesis of amoxicillin antibiotic. Penicillin-G Acylase (PGA), produced by microorganisms such as *Escherichia coli*[2], *Mucor griseocyanus*[3], *Bacillus megaterium*[4], *Achromobacter* sp[5], *Alcaligenes faecalis*[6], and *Kluyvera citrophila*[7], plays a vital role in catalysing the hydrolysis of Penicillin G to produce 6-amino penicillanic acid (6-APA), a key precursor for semi-synthetic penicillin production.[8] Enzyme immobilization has emerged as a promising strategy to reduce production costs, offering advantages such as increased stability against heat, pH variations, and harmful solvents, along with ease of separation from products.[9,10] Among immobilization methods, entrapment using alginate matrices has gained prominence due to its simplicity and cost-effectiveness.[11] The entrapment method is widely used in early studies of immobilized PGA. It involves encapsulating PGA within a carrier, which enhances the reuse capability of the immobilized enzyme by increasing diffusion resistance and allowing PGA to move over longer distances.[12]

Previous studies have contributed valuable insights into PGA immobilization strategies and their applications in pharmaceutical production. Immobilization of penicillin G acylase (PGA) has known able to stabilizing PGA and improving its catalytic activity as mention in Liu et al, while Jin et al. enhanced enzyme activity by immobilizing PGA using chitosan-resin.[14] Žuža et al. explored covalent immobilization of chemically modified PGA from *E. coli,* showing superior stability compared to conventional methods.[15] Additionally, Bernardino et al. achieved stable enzyme activity by immobilizing PGA from *E. coli* using nano-magnetic particles in a silica matrix.[16] Chen et al. used modified TiO_2 as a carrier to immobilize PGA, and the pH stability, thermal stability, and storage stability of the immobilized PGA were greatly improved compared to the free PGA.[17] Chen et al. reported that PGA immobilization through the reaction between the amino groups of PGA and the epoxy groups of paramagnetic polymer microspheres enhanced thermal and pH stability in extreme environments, making it a highly promising catalyst for industrial applications.[18] However, despite these advancements, there remains a gap in understanding the optimal conditions for PGA immobilization using less complicated method and material such as sodium alginate (Na-alginate) and its application in amoxicillin synthesis.

Building upon existing knowledge, our study aims to optimize the immobilization process, particularly through entrapment techniques using Na-alginate, and evaluate PGA stability under specific pH and temperature ranges. The immobilized PGA BD1 enzyme was derived from the *Bacillus thuringiensis* BD1 isolate, also referred to as *Bacillus thuringiensis* BGSC BD1,[19] sourced from the Bioindustrial Laboratory, BRIN Culture Collection, and recognized in previous studies for its demonstrated production of PGA.[18] We determined optimal conditions for immobilized PGA BD1 preparation by optimizing Na-alginate concentration and evaluated its activity and protein content through hydrolysis testing. Stability testing was performed within pH 6-9 and temperature 30-60 °C ranges, optimal for PGA BD1 production. The immobilized PGA BD1's activity influences amoxicillin production, making stability testing critical. UPLC synthesis testing assessed PGA BD1's ability to synthesize 6-APA into amoxicillin. SEM examined surface morphology of Na-alginate beads. Our study contributes to advancing pharmaceutical manufacturing, enhancing access to critical antibiotics like amoxicillin.

2. Materials and Methods

2.1. Materials

The research materials included Na-alginate (Himedia, India), Tris-Cl buffer pH 7 (Himedia, India), CaCl2 (Merck, Germany), PGA BD1 enzyme (produced using *Bacillus thuringiensis* BD1 isolate from Bioindustrial Laboratory, BRIN Culture Collection[19]), glutaraldehyde (Sigma Alrich, USA), chitosan HMW (Sigma Alrich, USA), and reagents for PGA activity analysis, namely PDAB (Merck, Germany), acetic Acid (Merck, Germany), penicillin-G (Sigma Alrich, USA), Tris-Cl buffer pH 7 1M, NaOH (Merck, Germany)), as well as reagents for PGA protein content analysis or Bradford reagent (Biorad, USA).

2.2. Methods

2.2.1. Production of PGA BD1 enzyme

The enzyme Penicillin G Acylase BD1 (PGA BD1) was produced using *Bacillus thuringiensis* BD1 isolate in a 5 L Erlenmeyer flask with standard LB media volume of 1 litre. The fermentation conditions included a 10%

starter volume, temperature of 37°C, agitation at 250 rpm, and pH 7 for a fermentation duration of 20 hours. Subsequently, downstream processing involved separating cell mass from the fermentation fluid using a refrigerated centrifuge (Tomy, Japan) at about 4°C. The obtained supernatant was then concentrated using filtration with an ultrafiltration membrane with a pore size of 10 kDa (Sartorius, Germany).

2.2.2. Immobilization in Na-Alginate

To immobilize the PGA BD1 enzyme within a Na-alginate matrix, Na-alginate solutions at concentrations of 1%, 1.25%, and 1.5% (w/v) were prepared in Tris-Cl buffer (pH 7.0) and heated to 50°C under stirring. These alginate concentrations were chosen based on previous studies on lipase enzyme immobilization, where a 1% alginate concentration resulted in the highest enzyme activity compared to 2% and 3% concentrations[20], which produced mechanically less stable matrices. Once fully dissolved, the solutions were cooled to room temperature. Subsequently, 2 mL of enzyme stock solution was combined with 8 mL of Na-alginate solution to achieve a final volume of 10 mL. The mixture was then transferred into a syringe. The solution was then dropped into a solution of 3 M $CaCl_2$ and 2.5% chitosan while gently stirring at 40°C for 2 hours. Glutaraldehyde (2.5%) was then added, and the mixture was stirred again for 1 hour.[21] The formed beads were filtered, washed with Milli-Q water, and dried with filter paper (Whatman-Merck, Germany).

2.2.3. Preparation of analysis reagents

The preparation of reagents for activity and protein content analysis of immobilized PGA BD1 enzyme included the preparation of Tris-Cl buffer pH 7, Penicillin-G 0.5 M, 4-dimethylamino benzaldehyde (PDAB) 1%, acetic acid 20%, and NaOH 50 mM solutions.

To prepare buffer Tris-Cl pH 7, Tris (12.11 g) was dissolved in 80 mL of distilled water and homogenized. The pH was adjusted to 7 using 1M HCl (Merck, Germany) and then the buffer was diluted with distilled water to a final volume of 100 mL. A solution of Penicillin-G 0.5 M (50 mL) was prepared by dissolving 9.31 g of Penicillin-G powder in Tris-Cl buffer pH 7 (0.05 M) and homogenizing it. Fresh Penicillin-G substrate was always prepared to avoid affecting activity measurement results. To obtain a solution of 4-dimethylamino benzaldehyde (PDAB) 1%, 0.2 g of PDAB was dissolved in 20 mL of methanol (Merck, Germany), homogenized, and stored in a Duran bottle lined with aluminium foil. Acetic acid 20% was prepared by mixing 20 mL of pure acetic acid with 80 mL of distilled water to obtain a 20% acetic acid solution. The solution was stored in a Duran bottle lined with aluminium foil. NaOH 50 mM was prepared by weighing 0.4 g of NaOH to be dissolved in 200 mL of distilled water. A stock solution of PDAB (3.5 mL) was prepared by mixing PDAB 1% (0.5 mL), acetic acid 20% (2 mL), and NaOH 50 mM (1 mL). The Bradford solution (Biorad Protein Assay reagent) was diluted 5 times using distilled water and then filtered using filter paper. The result was stored in a dark bottle lined with aluminium foil.

2.2.4. Analysis of immobilized PGA BD1 activity

PGA BD1 activity testing was performed by incubating 100 µL of Penicillin G substrate with 25 µL of enzyme sample at 50°C for 5 minutes. Afterward, 875 µL of 0.5% PDAB reagent was added, and the mixture was further incubated for 1,5 minutes, followed by absorbance measurement at a wavelength of 415 nm. Blank measurements were performed by incubating 25 µL of enzyme at 50°C for 5 minutes, followed by the addition of 875 µL of 0.5% PDAB reagent and 1-minute incubation. The mixture was then added to 100 µL of Penicillin G substrate and incubated for 1 minute, followed by absorbance measurement at a wavelength of 415 nm.[22] The subsequent readings were converted into the standard linear equation of 6-APA (Sigma Alrich, USA):

$$PGA\ activity\left(\frac{U}{mL}\right) = \frac{[6-APA] \times 1000}{(MW \times t \times Ve)}$$

Where [6-APA] represents the concentration of 6-APA produced, 1000 is the conversion factor in µmol, MW is the molecular weight of Penicillin G (216.25), t is the incubation time at the optimal temperature (5 minutes), and Ve is the volume of crude enzyme extract used (25 µL = 0.025 mL).

2.2.5. Protein content analysis

Protein content was determined using the Bradford method with BSA (Bovine Serum Albumin (Himedia, India)) as the standard protein. Standard solutions were prepared using a BSA stock solution of 1 mg/mL. Samples were mixed with Bradford reagent, and after 10 minutes of incubation at room temperature, the absorbance was measured at 595 nm wavelength.

2.2.6. pH and temperature stability test

Optimization of pH stability (pH 6-9) was conducted at 37°C, the standard temperature for PGA activity measurement during production. Subsequent testing involved stability assessment at temperatures ranging from 30 to 60°C, performed at the optimum pH for production to evaluate the stability of immobilized PGA BD1 at each temperature.

2.2.7. Reusability test

The reusability test of immobilized PGA BD1 involved measuring the enzyme activity using the Balasingham method[22] after reaction with the substrate. After washing the enzyme with Tris-Cl buffer pH 7, enzyme activity was measured using the same method as the initial test. Three tests were conducted, each with four repetitions.

2.2.8. Amoxicillin synthesis test

Amoxicillin was synthesized from 6-APA and HPGME (Sigma Alrich, USA) using immobilized Penicillin G Acylase (PGA BD1), and the synthetic enzyme activity was calculated based on the amount of amoxicillin produced. A solution of 6-APA at 20 mM was prepared by weighing 0.43 g of 6-APA and adding approximately 50 mL of water. The pH was adjusted to 7.5-7.6 using 6 mol/L ammonia, and then the solution was dissolved. Additional water was added to reach a constant volume of 100 mL. HPGME at 60 mM was prepared by weighing 1.30 g of d-hpgm (Sigma Alrich, USA) and adding approximately 50 mL of water. The pH was adjusted to 2.9-3.0 using 6 M HCl, and then the solution was dissolved. Additional water was added to reach a volume of 100 mL. Immobilized PGA BD1 enzyme pretreatment involved rinsing with purified water 8-10 times until the water became clear, followed by filtration with a glass funnel until no droplets flowed out.

A mixture of 100 mL of 6-APA solution was added to an Erlenmeyer flask at a temperature of 25.0°C. Then, 100 mL of HPGME solution and 1 g of pretreated enzyme sample were added. The pH was adjusted to 6.3. The clear solution from the reaction mixture was collected using a 5 mL syringe with a filter to determine amoxicillin concentration using Ultra Performance Liquid Chromatography (UPLC).

2.2.9. Surface morphology analysis

Surface morphology of the beads formed from Na-alginate was examined using Scanning Electron Microscopy (SEM). The beads were coated with gold using ion sputtering and observed using a scanning electron microscope at the Advanced Characterization Laboratory, BRIN Gunung Kidul Playen, Indonesia.

3. Result

3.1. Penicillin G Acylase production

In this study, the production of PGA BD1 enzyme was conducted utilizing the Bacillus thuringiensis BD1 isolate. The measured enzyme activity was 2.63 U/mL, with a specific activity of 46.59 U/mg. This yielded PGA BD1, which was subsequently employed in optimizing PGA BD1 enzyme immobilization using Na-alginate.

3.2. PGA BD1 enzyme in Na-Alginate immobilization matrix

Analysis of PGA BD1 activity post-immobilization revealed a decline compared to the free or pre-immobilization PGA BD1 enzyme (Figure 1). Optimal specific activity was attained at a Na-alginate concentration of 1.5% (41.01 U/mg). At lower Na-alginate concentrations (1% and 1.25%), approximately a 50% reduction in activity was observed compared to pre-immobilization enzyme activity.

3.3. Stability test of immobilized PGA BD1

Stability tests revealed optimal activity at pH 7 (41.21 U/mg), signifying peak activity at neutral pH, consistent with PGA production and hydrolysis activity analysis. Optimal performance was maintained within pH 6-8, with a slight activity decrease observed at pH 6 and pH 8 (Figure 1). Conversely, pH 9 induced a significant activity decrease.

Temperature stability tests indicated sustained high activity between 30 °C and 60 °C, with optimal performance achieved at 40 °C within a 5-minute incubation period (Figure 2). However, activity decreased at higher temperatures (50-60°C). Collectively, PGA BD1 immobilization displayed

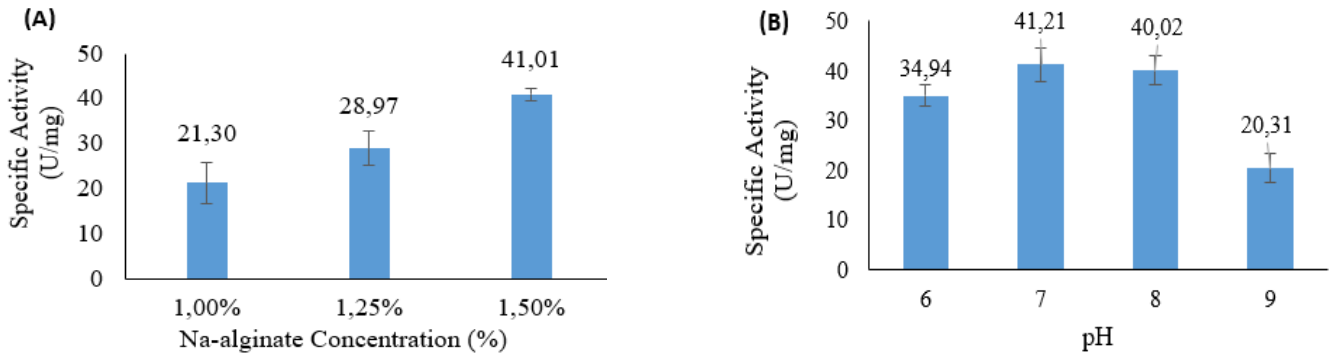

Figure 1. Effect of Na-alginate concentration (A) and pH stability test (B) of the immobilized PGA BD1

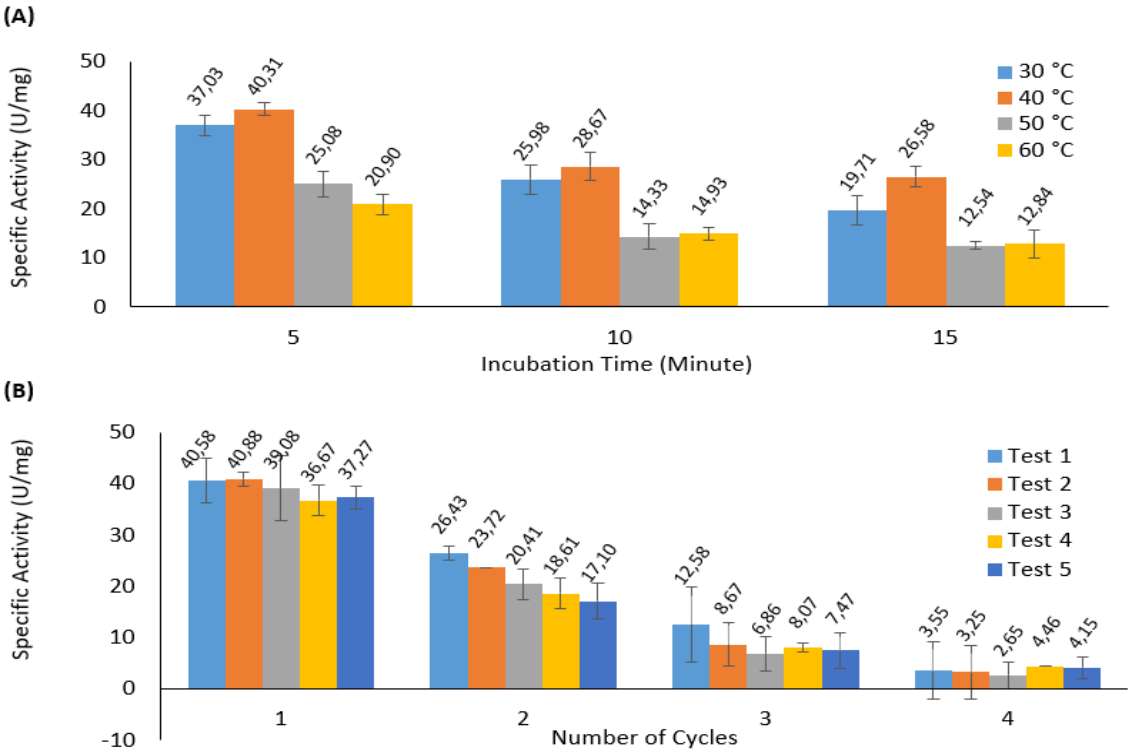

Figure 2. Temperature Stability Test (A) and Reusability Test (B) of the immobilized PGA

characteristic activity within a favourable temperature range of approximately 30-60 °C.

3.4. Immobilized PGA BD1 Reusability Test

The result of the reusability test of PGA BD1 immobilized with Na-alginate showed a decline in activity after successive use of the immobilized enzyme in reactions (Figure 2). Following three repetitions of the test, it was evident that the immobilized PGA BD1 enzyme retained a certain level of activity for up to 4 uses. The decrease in enzyme activity after the second use averaged approximately 65%. Subsequently, in the third use, the immobilized enzyme retained activity at around 40%. After approximately 4 uses, only about 20% of the initial activity remained.

3.5. Immobilized PGA BD1 Morphology test

The SEM analysis reveals disparities between the images of alginate matrices pre- and post-immobilization (Figure 3). The SEM images reveal distinct differences between Na-alginate matrix without immobilized PGA BD1 (Figure 3, left) and PGA BD1 immobilized on Na-alginate (Figure 3, right). The unmodified Na-alginate exhibits a loose and porous structure with larger, irregular pores, indicative of its natural state. In contrast, the immobilized sample demonstrates a more compact and organized microstructure, with smaller and uniformly distributed pores. This structural transformation suggests that the immobilization process enhances the material's density and mechanical stability, likely improving its suitability for enzyme immobilization and biocatalytic applications.

3.6. Amoxicillin synthesis test

Results comparing amoxicillin concentration from reactions using immobilized and free PGA BD1

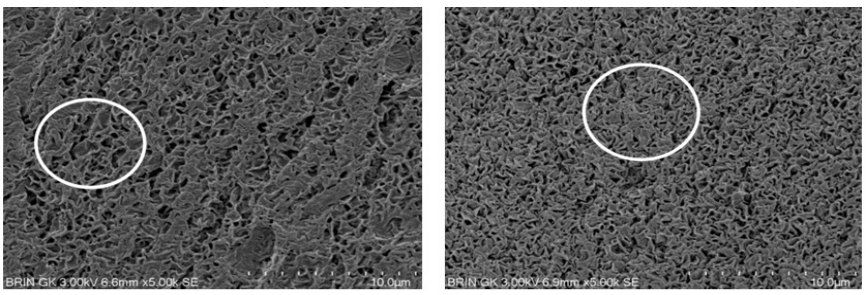

Figure 3. Immobilized PGA BD1 morphology: Na-Alginate without (left) and with (right) immobilized PGA BD1

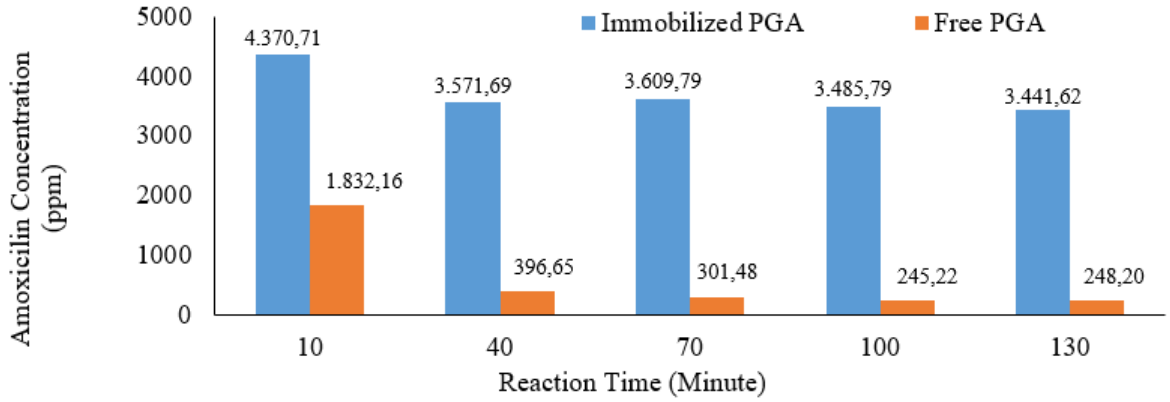

Figure 4. Amoxicillin synthesis test

demonstrates that PGA BD1 immobilized with Na-Alginate yields a higher concentration of amoxicillin than its free form (Figure 4).

4. Discussion

In this study, the measured activity of the Penicillin-G Acylase (PGA) DB1 enzyme was 2.63 U/mL, with a specific activity of 46.59 U/mg. Comparable studies have shown optimal PGA BD1 production in the range of 3 U/mL.[19] Post-immobilization analysis of PGA BD1 indicated a decline in activity compared to the free enzyme, likely due to diffusional limitations that hinder catalytic activity.[23]

Optimal specific activity was observed at 1.5% Na-alginate concentration (41.01 U/mg), where low gel porosity facilitated optimal enzyme entrapment.[24] During preliminary experiments, Na-alginate concentrations above 1.5% (w/v) resulted in solutions that were excessively viscous and dense, making bead formation via syringe extrusion highly challenging due to clogging. The elevated viscosity likely hindered uniform enzyme distribution within the alginate matrix, potentially compromising the immobilization efficiency. Furthermore, beads formed at higher alginate concentrations were harder and more brittle, raising concerns about their mechanical stability during practical applications. Increased matrix density might also restrict enzyme-substrate interactions, thereby reducing the catalytic activity of the immobilized enzyme. Lower concentrations (1% and 1.25%) resulted in about a 50% reduction in activity due to increased enzyme leakage, while higher concentrations led to denser matrices, impeding diffusion and reducing activity.[20]

The instability and lack of reusability of free enzymes, and the challenge of separating the enzyme from the product are some of the limiting constraints affecting the industrial application of Penicillin G acylase (PGA).[25] Stability tests across pH and temperature gradients were crucial in determining their impact on the activity and stability of immobilized Penicillin-G Acylase (PGA) from *B. thuringiensis* BD1. These tests help optimize conditions to enhance enzyme performance in various applications. PGA BD1 showed optimal performance within pH 6-8, with slight activity decreases at pH 6 and pH 8, and a significant decrease at pH 9 due to reduced active site formation and structural denaturation. Temperature stability tests revealed sustained high activity between 30°C and 60°C, with optimal performance at 40°C after 5 minutes, though activity decreased at higher temperatures due to thermal denaturation, consistent with previous studies.[12] Immobilized PGA BD1 demonstrated stable activity within 30-60°C, corroborating Mayer et al.'s findings of a melting point around 50-60 °C.[26]

The success of the immobilization technique depends on the immobilized enzyme's ability to sustain multiple reactions without significant activity loss. The reusability test of PGA BD1 immobilized with Na-alginate demonstrated a decline in activity with repeated use. After three cycles, the immobilized enzyme maintained about 20% of its initial activity after the fourth use, decreasing to approximately 65% after the second use and around 40% after the third use. This decline is attributed to structural damage to the enzyme and matrix from repeated use, including physical and chemical stress, substrate interactions, and product contact27. Matrix damage may result in structural changes or surface property loss, impairing enzyme performance and stability. Enzyme loss from the alginate matrix and the accumulation of side products can further impede enzyme access to the matrix pores.

Scanning Electron Microscopy (SEM) analysis compared the morphology of Na-Alginate beads with and without immobilized PGA BD1, revealing differences pre- and post-immobilization. The entrapment method often results in uneven enzyme distribution within the alginate matrix, leading to

random dispersion or clustering and variations in porosity. Enzymes filling matrix interstices can reduce pore size, consistent with observations by Nawaz et al., who noted empty cavities pre-immobilization and irregular particle localization post-immobilization.[28]

Amoxicillin synthesis was analysed using Ultra Performance Liquid Chromatography (UPLC). The reaction of 6-APA and HPGME with Na-Alginate immobilized PGA BD1 showed higher amoxicillin concentrations compared to reactions with free enzymes. This aligns with studies like Ro'yal Aini and Ratnayani, where immobilized enzymes outperformed free forms.[24] Enhanced enzyme-substrate interaction likely accelerates reaction rates, leading to higher product yields. Immobilized enzymes offer greater stability, protecting against deactivation and prolonging efficacy, crucial for higher amoxicillin production.[29]

Immobilized Penicillin-G Acylase (PGA) enzymes exhibit superior stability, maintaining their activity over longer durations. The interaction between the enzyme and the immobilization matrix helps preserve reaction substrates, thereby improving reaction efficiency.[30]

5. Conclusion

The investigation into Penicillin-G Acylase (PGA) enzyme immobilization revealed key insights. Post-immobilization, the enzyme's specific activity decreased from 46.59 U/mg to an optimal 41.01 U/mg at a Na-Alginate concentration of 1.5%. The concentration of alginate significantly influenced the enzyme-substrate binding affinity. Stability tests identified peak enzyme activity at pH 7 and 40°C, aligning with optimal conditions for PGA BD1 production. The enzyme remained functional within a temperature range of 30-60°C. Repeated usage of the immobilized enzyme showed about 20% residual activity after four uses, likely due to structural damage. SEM imaging indicated an even distribution of enzymes within the alginate matrix. Notably, PGA BD1 immobilized with Na-Alginate demonstrated superior amoxicillin synthesis compared to its free form, yielding higher amoxicillin concentrations. This study underscores the potential of Na-Alginate immobilized PGA BD1 in enhancing amoxicillin production efficiency.

6. Author Contributions

R. A. P. Dewi, E. Widyasti, and Dianursanti, designed the research; R. A. P. Dewi, S. Kusumaningrum, M. D. Rahayu, N. L. Putra, N. F. Hasanah, R. G. Sativa, and D. Nandyawati performed the experiments and provided the data; R. A. P. Dewi analysed and interpreted the data; R. A. P. Dewi, and C. Sriherwanto wrote, reviewed, and edited the manuscript; E. Widyasti, Dianursanti, and S. Setyahadi supervised the research; E. Widyasti additionally undertook funding acquisition.

Conflict of Interest

The authors declare no conflicts of interest.

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