

Improvement of Thermo-Stability and Solvent Tolerant Property of *Streptomyces sp.* A3301 Lipase by Immobilization Techniques with Application in Poly (lactic acid) Polymerization by Using Biological Process

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Abstract

The thermo-solvent-tolerant lipase-producing actinomycete, *Streptomyces sp.* A3301, was utilized as a biocatalyst for poly (lactic acid) or PLA polymerization. The study aimed to optimize lipase immobilization conditions, characterize the immobilized lipase and apply it for PLA polymerization. The results showed using a sponge as the immobilizing matrix was the most effective method, achieving a maximum activity of 277 U/g of sponge. The optimal sponge size was determined to be 0.125 cm³ and pre-soaking the sponge in 0.1 M phosphate buffer at pH 7.0 for 24 h before use proved advantageous. Immobilization significantly enhanced the thermo-stability of the enzyme, with a relative activity ranging from 140 to 190 % within the temperature range of 30 to 60 °C. In contrast, the crude lipase exhibited thermo-stability only within the 30 - 50 °C range. The immobilized lipase demonstrated stability under PLA polymerization conditions, which involved a reaction mixture containing toluene and lactic acid and performed at 60 °C for 8 h. The immobilized lipase maintained its activity under this condition for 5 h, retaining a relative activity of 230 %, which was 1.2 times higher than the activity of the crude lipase. When the immobilized lipase was used in PLA polymerization, the resulting PLA product exhibited a molecular weight of 5,333 ± 0.02 Da, and the degree of polymerization was approximately 72. These findings underscore the potential of the immobilization technique to enhance lipase activity for PLA polymerization.

Keywords: Poly (lactic acid) or (PLA), Lipase, Enzyme immobilization, Sponge, Adsorption method, Thermo-solvent tolerant lipase, PLA -polymerization, Biological process

Introduction

Plastic waste has become a significant global issue due to the extensive use of plastics in everyday human life. The complex structure of plastics and their slow natural degradation process have led to adverse environmental impacts, affecting both non-living and living organisms. This environmental pollution is exacerbated by disposal methods such as burial and incineration, with contribute to global warming.

Therefore, biodegradable plastics have gained popularity as a promising alternative to conventional petroleum-based plastics. Biodegradable plastics, including poly (lactic acid) or PLA, can naturally degrade and have minimal environmental impact. Several types of biodegradable plastics, such as poly (lactic acid) (PLA), poly (β -hydroxybutyrate) (PHB), and poly (ϵ -caprolactone) (PCL), have been widely studied and applied to replace conventional plastics derived from petrochemicals.

In this context, PLA, a biodegradable plastic synthesized from lactic acid, has attracted considerable attention. Lactic acid can be obtained through fermentation processes using agricultural residues like sugarcane, corn, and rice. The synthesis of poly (lactic acid) generally involves chemical methods, such as direct polycondensation reaction and ring-opening polymerization. However, these chemical methods often require high temperatures, concentrated solvents, and accelerators, leading to extreme reaction conditions. In this study, researchers have explored the use of enzymes from microorganisms to catalyze the synthesis of biodegradable PLA, offering a more environmentally friendly and milder alternative [1-3].

In the previous study, a biological approach was used to enhance the properties of enzyme catalysts for PLA synthesis. Specifically, enzymes from microorganisms that can withstand high temperatures and organic solvents were selected. The enzyme from *Streptomyces sp.* A3301 was identified as a suitable catalyst for PLA synthesis. This enzyme could catalyze the synthesis of PLA with a molecular weight of 525 Da and a degree of polymerization of 7 at an optimal temperature of 60 °C for 8 h. However, during the synthesis process, the enzyme activity could be compromised by high temperatures and toluene as organic solvents, leading to reduced efficiency and a decrease in the polymerization rate [4].

In this study, interesting 3 enzyme immobilization techniques such as adsorption, encapsulation, and cross-linking. The immobilization techniques were explored to improve the enzyme stability and performance. Immobilization involves attaching the enzyme to an immobilization material, reducing direct contact with the organic solvent, and protecting the enzyme from high temperatures [5]. The study aims to develop immobilization methods suitable for the enzyme from *Streptomyces sp.* A3301 and compare the catalytic efficiency of immobilized enzymes with crude enzymes and optimize enzyme immobilization techniques for enhancing the synthesis of biodegradable PLA. The study focused on developing a sustainable and environmentally friendly approach to PLA synthesis by utilizing enzyme catalysts. The findings contribute to the potential application of biologically catalyzed PLA synthesis as an alternative to traditional chemical methods, offering a more efficient and eco-friendlier pathway to produce biodegradable plastics.

Materials and methods

Microorganism

The cultivation of *Streptomyces sp.* A3301, isolated through the screening conducted by Panyachanakul *et al.* [4] was carried out using International Streptomyces Project Medium 2 (ISP2 medium) as the growth substrate. The strain was incubated at room temperature for 7 days. Subsequently, 1 loop of it was transferred to ISP2 broth and incubated at 30 °C under agitation at a speed of 150 rpm for 3 days. The culture was used as the inoculum for enzyme production.

Lipase production

The inoculum, 10 % (v/v), was inoculated into the production medium was modified by [4] (composed of 1.5 % sucrose, 2 % yeast extract, and 0.1 g/L Na_2HPO_4) and incubation at 30 °C under agitation at a speed of 150 rpm for a duration of 3 days. Following this, the enzyme was harvested using a centrifugation

method carried out at 4 °C and 10,000 rpm for 20 min. The resulting supernatant was separated from the cell pellet and employed as the crude enzyme solution for further use.

Optimization of lipase immobilization

This research thus chose to employ the adsorption method for enzyme immobilization. The adsorption method is a straightforward approach for immobilization using a carrier-bound technique. This technique relies on physical adsorption, which involves weak binding forces such as hydrogen bonds, ionic bonds, and hydrophobic bonds between the carrier and the enzyme. This method is cost-effective and easy to implement [5]. The adsorption method is using a suitable immobilization material known as scrub pad (Scotch-Brite3M). Scrub pad is derived from synthetic materials such as cellulose, nylon, and polypropylene, which adhere to enzymes through ionic or van der Waals interactions between the enzymes and scrub pad, as well as through sponge (Scotch-Brite3M) made from cellulose, which attach to the enzyme using ionic or van der Waals interactions between the enzyme and the cellulose sponge. Cellulose-based immobilization materials are commonly used to immobilize enzymes such as amylase, trypsin, lipase, and beta-galactosidase [5,6]. The encapsulation method involves entrapping several biomolecules such as enzymes or cells into different polymeric matrices [5]. The encapsulation method was also employed, using an immobilization material such as calcium alginate. Calcium alginate beads are derived from the cell walls of brown algae, with alginate obtained from the cell wall of brown algae. Calcium chloride solution is used to solidify the alginate beads. This method enhances enzyme activity and allows for enzyme reuse. Moreover, Cross-linking immobilization is a strategy that involves interconnecting enzymes through covalent bonding, without the need for carriers. This intermolecular cross-linking is achieved with the help of linker agents, which serve as bridges between 2 adjacent enzyme molecules [5]. This study, use of gelatin and starch as cross-linked immobilization materials involves the use of natural polysaccharide polyacrylamide and the addition of glutaraldehyde to create bonds between the enzyme molecule and the immobilization material, thereby increasing the enzyme's stability [5,6].

Effect of immobilization material and immobilization method on lipase activity

The enzyme lipase was immobilized using various immobilization materials and different methods. The adsorption method was done by adding 10 g of scrub pad and sponge (1 cm³) in a solution containing 100 mL of crude lipase enzyme. Additionally, encapsulation was done by A 10 mL enzyme solution is mixed with 2 % calcium alginate. This mixture is then dropped into 20 mM Calcium chloride solution, cross-linking method was done by A 10 mL enzyme solution is mixed with 10 % gelatin and 10 % glutaraldehyde. This mixture is then poured into a sterilized tray and cut into cubes of 1 cm³ each, and A 10 mL enzyme solution is mixed 0.1 % starch and 1.5 % glutaraldehyde. This mixture is then poured into a sterilized tray and cut into cubes of 1 cm³ each [5]. That were used as an immobilization material. The enzyme mixture was immobilized at 4 °C for 24 h. Afterward, the immobilized enzymes were washed with 0.85 % NaCl solution, and the immobilization method and material were selected based on the enzyme activity on the immobilization material (U/g of immobilization material) and the experiment was done in triplicate.

Effect of temperature and toluene on lipase activity

The effect of temperature on the enzyme activity of immobilized lipase and its tolerant to toluene were also studied, for thermo-stability was investigated by pre-incubated 1 g of the immobilized enzyme

on various immobilization material at 60 °C for 1 h and for toluene tolerant was studied by pre-incubated 1 g of the immobilized enzyme in 1 mL of toluene for 1 h at 30 °C. After incubating, washed twice with distilled water, and determined relative activity and the experiment was done in triplicate.

Effect of pH on lipase activity

The sponge was soaked in a buffer solution with a pH range of 4.0 to 9.0 (Acetate buffer pH 4.0 - 5.0, Phosphate buffer pH 6.0 - 9.0 and Glycine-NaOH buffer pH 9.0) at 4 °C for 24 h prior to the immobilization process. After that, 10 g of the sponge was soaked in 100 mL of crude lipase solution at a temperature of 4 °C for a duration of 24 h. After the immobilization period, the enzyme activity was determined, and the experiment was done in triplicate.

Effect of size of immobilization material on lipase activity

The study determined that sponge is the most effective immobilization material for immobilizing lipase. The size of the sponge used for immobilization was tested at 2 different sizes: 0.125 and 1 cm³. For the immobilization process, 10 g of the sponge was soaked in 100 mL of crude lipase solution at a temperature of 4 °C for a duration of 24 h. After the immobilization period, the enzyme activity was determined to assess the effectiveness of the immobilization process using the selected immobilization material and size and the experiment was done in triplicate.

Effect of incubation time on lipase activity

The research concluded that the optimal size of the sponge for immobilization is 0.125 cm³. Following this determination, the sponge was soaked in 100 mL of crude lipase. The experiment was conducted over a period of 84 h, with sampling intervals of 12 h and determined the enzyme activity and the experiment was done in triplicate.

Assay of lipase activity

The lipase activity was determined by a spectrophotometric assay using *p*-nitrophenyl palmitate (*p*NPP) as a substrate. The reaction mixture consisted of 1 g of immobilized enzyme and 1 mL of substrate (3 mg of *p*NPP dissolved in 1 mL of isopropanol and 9 mL of 0.1 M phosphate buffer, pH 7.0). The reaction mixture was incubated at 37 °C for 30 min and then boiled in water for 5 min; then, 5 mL of distilled water was added to stop the reaction and determined at the optical density 410 nm. *p*-nitrophenol was used as a standard over a concentration range of 20 - 200 µM/mL. One unit of lipase activity was defined as the amount of enzyme that liberated 1 µM of *p*-nitrophenol per minute under assay conditions. The enzyme activity method was modified from [4].

$$\text{Relative activity} = \frac{\text{Enzyme activity of sample} \times 100}{\text{Enzyme activity of control}}$$

Characterization of enzyme lipase for PLA-polymerization

Effect of temperature on lipase activity

The thermo-tolerant of lipase was investigated by immobilized lipase were subjected to incubation at various temperatures ranging from 30 to 80 °C for a duration of 60 min, prior to the analysis of the relative lipase activity. A comparative study was conducted against crude enzyme as control and the experiment was done in triplicate.

Efficiency of immobilized-lipase under PLA-polymerization condition

The immobilized enzyme on the optimal immobilization material, its efficiency in withstanding conditions utilized in the synthesis of poly (lactic acid), was assessed. In the reaction mixture consisting of 450 g/L of lactic acid as the substrate, 55 % (v/v) of toluene as the solvent, and 10 % (w/v) of the immobilized enzyme was employed, the reaction was conducted at a temperature of 60 °C, maintaining samples at hourly intervals for a total duration of 8 h [4]. Following the reaction, post-separation of the immobilized enzyme from the reaction mixture, it was subjected to 2 consecutive rinses with distilled water, and subsequently analyzed for the relative activity of the immobilized enzyme and the experiment was done in triplicate.

Application of immobilized-lipase on PLA-polymerization

Preparation of immobilized lipase

The enzyme lipase was immobilized onto a suitable immobilization matrix, following the appropriate conditions. Subsequently, the immobilized lipase samples were subjected to remove water from the sample by using a freeze-drying process. This process involved freezing the immobilized enzyme at -80 °C for a duration of 48 h. Afterward, the immobilized lipase was dried using a freeze-drying apparatus for a period of 5 days. Following this procedure, the resulting dried immobilized lipase was employed as a catalyst in the synthesis of poly (lactic acid) in the polymerization process.

PLA-polymerization

The reaction mixture, composed of commercial-grade lactic acid at a concentration of 450 g/L as the starting material. Toluene was employed as the solvent for dissolution, and then, 0.5 g of immobilized lipase enzyme were incorporated. The enzyme exhibited an activity of 277 U/g. The reaction was conducted at a temperature of 60 °C, with continuous stirring throughout the process, in an ambient atmosphere, aided by the introduction of nitrogen gas. The reaction proceeded for a duration of 8 h. After completing the reaction, the removal of toluene was carried out through an evaporation process using an Evaporator at 60 °C. Following this, the poly (lactic acid) was extracted by adding 50 mL of dichloromethane solvent. The resulting mixture was then subjected to agitation at 150 revolutions per minute for 1 h. This process allowed for the separation of the dichloromethane solvent from the reaction mixture. The solvent phase containing dichloromethane was then evaporated using an Evaporator at 60 °C until complete evaporation was achieved, and the experiment was done in triplicate.

Subsequently, the plastic pellet was detached from the round-bottom flask using a 2 mL of tetrahydrofuran (THF) solvent. The solution was then filtered through a 0.22 µM filter to remove any impurities. The resulting solution was then ready for analysis, particularly to determine the molecular weight of the poly (lactic acid) by HPLC.

Molecular weight of PLA analysis

The molecular weight of the synthesized poly (lactic acid) can be analyzed using the technique of high-performance liquid chromatography (HPLC) with a Gel Permeation Chromatography (GPC) column, specifically the TSK-GEL HXL Series. The mobile phase for this analysis is tetrahydrofuran (THF) at a flow rate of 1 mL per minute, at a temperature of 30 °C. Detection is carried out at a wavelength of 254 nm. A standard polymer, polystyrene with a molecular weight range of 269 - 9320 Da, is used as a reference.

The degree of polymerization (DP) can be calculated using the formula:

$$\text{Degree of Polymerization} = \frac{\text{Total Molecular Weight of Polymer}}{\text{Molecular Weight of Monomer}}$$

Results and discussion

Optimization of lipase immobilization

Effect of immobilization material on lipase activity

The investigation into 3 enzyme immobilization methods, namely adsorption with sponge and scrub pad, encapsulation with calcium alginate bead and cross-linked with gelatin and starch, as well as the selection of 5 types of enzyme immobilization materials. The results have revealed that immobilizing enzymes through the adsorption method exhibited the highest enzyme activity. Subsequently, the entrapment method followed, and then the encapsulation method, in that order. Among these, the use of sponge as the best immobilization material yielded showed the highest enzyme activity, measuring 57.31 U/g of immobilization material. Following this, scrub pad was employed, resulting in an enzyme activity of 20 U/g of immobilization material for the immobilized enzyme. This value was notably higher than the enzyme immobilization using gelatin and starch, which was approximately 4 times lower, as shown in **Figure 1**. The optimization of various parameters on lipase immobilization, Effect of immobilization materials, pH, size of immobilization materials and incubation periods on lipase activity.

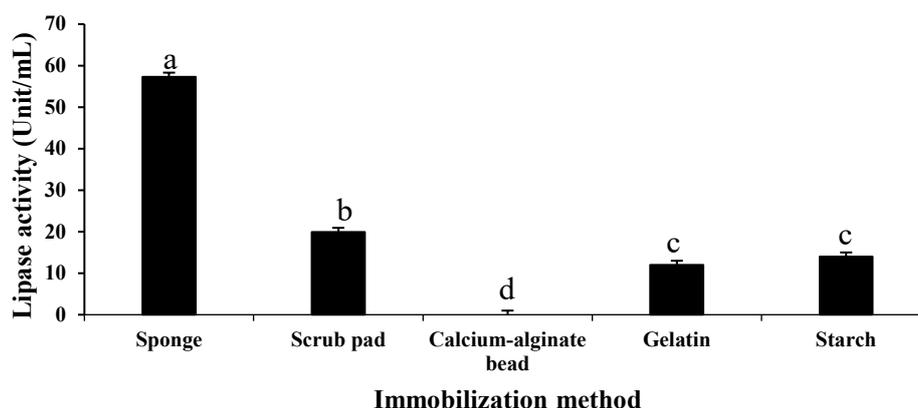


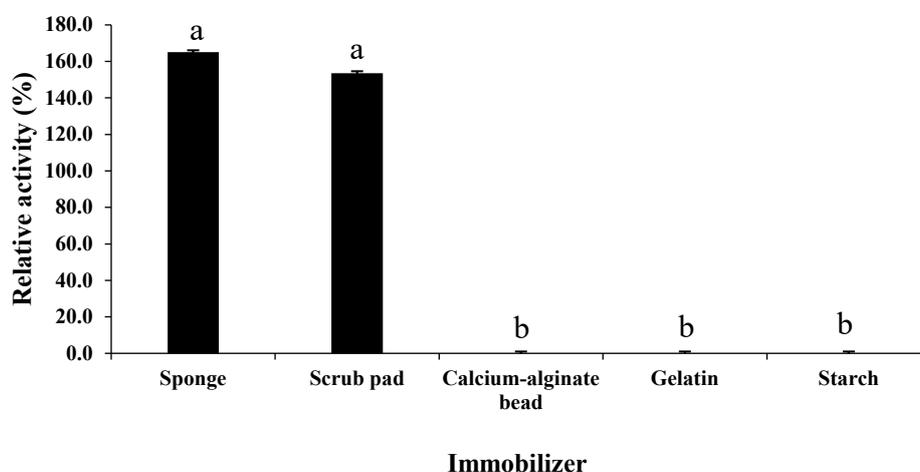
Figure 1 Effect of immobilization material and immobilization methods on lipase activity.

Effect of temperature on lipase immobilization

After studying the effects of various enzyme immobilization materials, including sponge, scrub pad, calcium alginate, gelatin, and starch, on enzyme activity, the investigation extended to examining the efficacy of utilizing these immobilization materials for the synthesis of biopolymer, specifically poly (lactic acid), at a temperature of 60 °C. Consequently, it became imperative to investigate the effect of temperature on the activity of the immobilized enzyme at 60 °C.

When the enzyme-immobilization materials were incubated at 60 °C for 1 h, the physical changes were observed in the immobilization materials calcium alginate, gelatin, and starch, which dissolved at this temperature. Thus, their enzyme activity could not be evaluated. Conversely, the enzymes immobilized on sponge and scrub pad displayed relative activities of 165 and 154 %, respectively, as depicted in **Figure 2**. Notably, the physical characteristics of the immobilization materials remained unchanged. As a result, both

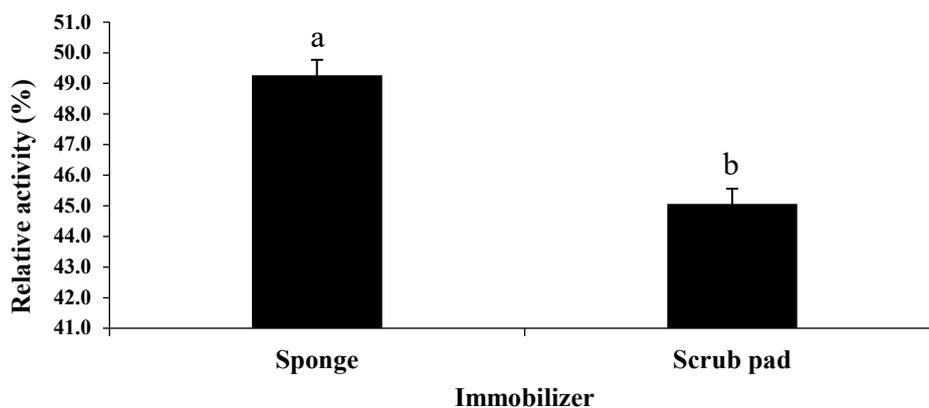
sponge and scrub pad were chosen as immobilization materials for the subsequent investigation of the effect of toluene on the activity of the immobilized enzyme.



Effect of toluene on lipase immobilization

The study of the temperature effects on the activity of immobilized lipase enzyme on various immobilization materials, it was found that only sponge and scrub pad were able to withstand a temperature of 60 °C. Consequently, we proceeded to investigate the impact of toluene on the activity of the immobilized lipase enzyme on sponge and scrub pad, as well as the physical characteristics of the immobilized materials.

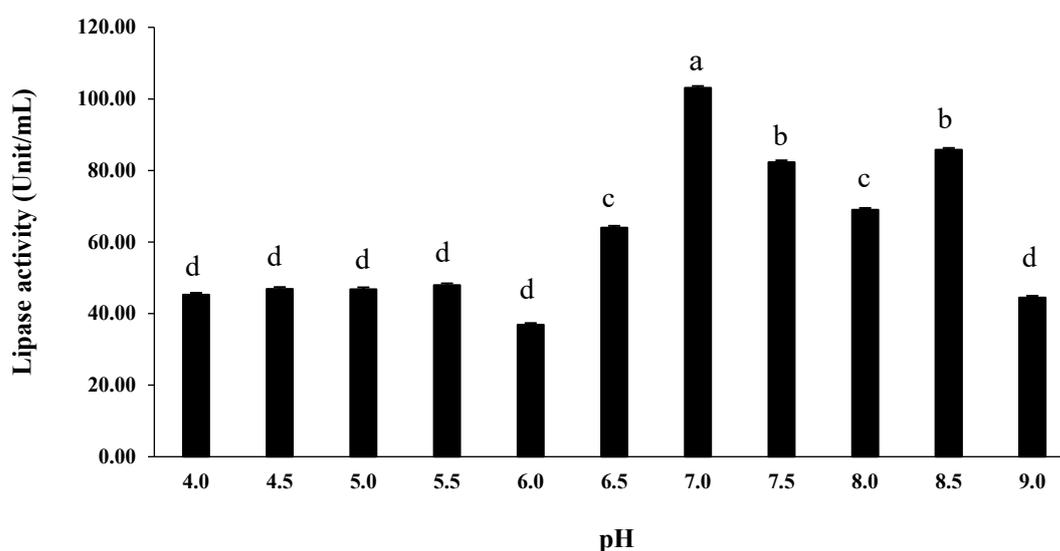
Experimental results revealed that when the lipase enzyme immobilized on sponge and scrub pad was incubated with toluene for 1 h, the relative activity values were 49.3 and 45.1 %, respectively, as shown in **Figure 3**. The physical characteristics of the immobilization materials remained unaltered. Therefore, within this research, sponge was chosen as the enzyme immobilization material due to its high temperature resistance and compatibility with toluene. Furthermore, this choice led to the highest relative activity of 57.31 U/g of immobilization material.



Effect of pH on lipase immobilization

Based on the study of enzyme immobilization methods and the selection of immobilization materials, sponge emerged as the most suitable enzyme immobilization material. Consequently, the focus shifted to enhancing the efficiency of enzyme immobilization, particularly concerning sponge as the adsorption-based immobilization material. sponge, derived from cellulose, utilizes hydrogen bonding, or van der Waals interactions to immobilize the enzyme onto its surface. This prompted an interest in augmenting the adhesive capacity of sponge to enhance enzyme adhesion.

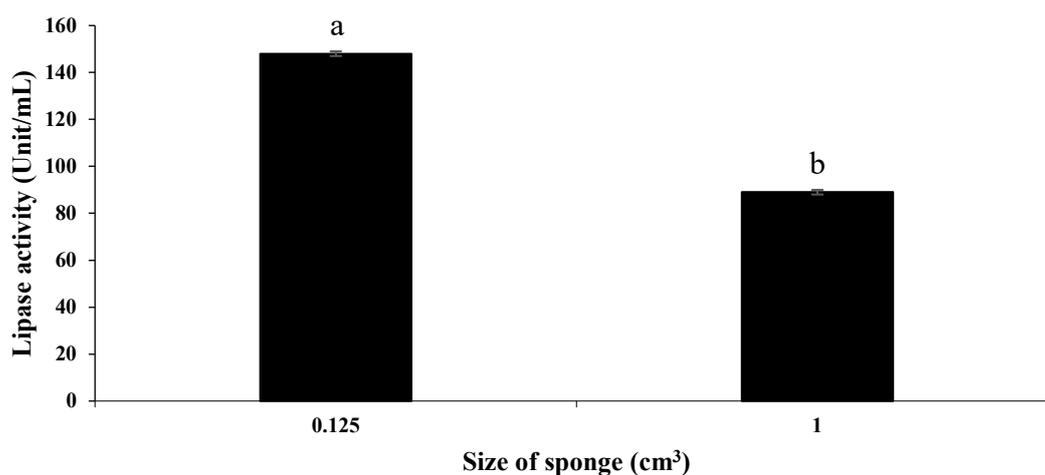
In pursuit of this, the sponge was soaked in a 0.1M phosphate buffer solution with a pH range of 4.0 to 9.0 at 4 °C for 24 h prior to the immobilization process. Experimental results demonstrated that the highest enzyme activity reached 103 U/g of immobilization material when the sponge was soaked in a phosphate buffer solution with a pH of 7.0 before enzyme immobilization, as shown in **Figure 4**. The outcomes indicate that pre-soaking the sponge in a phosphate buffer solution at pH 7.0 prior to immobilization led to significantly higher enzyme activity compared to using untreated sponge. This enhancement was approximately 2-fold, illustrating the influence of buffer pH on the adhesive interaction between the enzyme and the sponge immobilization material.



Effect of size of immobilization material on lipase activity

Studying the impact of the size of the immobilization material on enzyme activity, the investigation focused on the effect of sponge size on the activity of the lipase enzyme. Two sizes of sponge were studied, namely 0.125 and 1 cm³, in relation to their influence on enzyme activity.

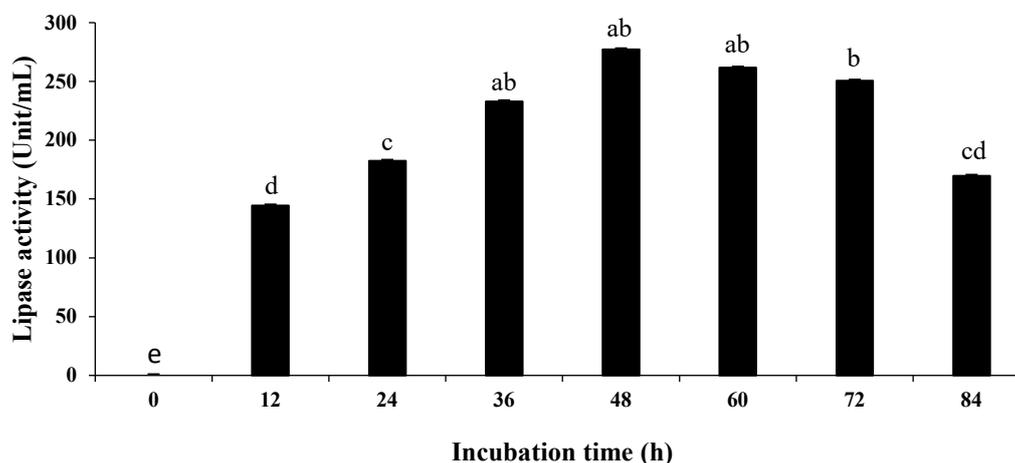
Experimental findings revealed that sponge with a size of 0.125 cm³ exhibited an enzyme activity of 148 U/g of immobilization material, whereas sponge with a size of 1 cm³ displayed an enzyme activity of 89 U/g of immobilization material, as illustrated in **Figure 5**. Consequently, within this research, sponge with a size of 0.125 cm³ was chosen as the enzyme immobilization material due to its higher enzyme activity compared to the larger sponge size.



Effect of incubation time on lipase activity

Investigating the influence of incubation time of sponge in the enzyme solution on enzyme activity, an experiment was conducted where sponge was left in the enzyme solution for a range of time periods, from 0 to 84 h. Subsequently, the enzyme activity was analyzed.

The experimental outcomes revealed that the highest enzyme activity of 277 U/g of immobilization material was achieved after soaking the sponge in the enzyme solution for a duration of 48 h. This value was higher than the enzyme activity obtained from soaking the sponge for 24 h, which was approximately 1.5 times. Furthermore, extending the soaking duration in the enzyme solution up to 84 h resulted in a reduction in enzyme activity to 170 U/g of immobilization material as shown in **Figure 6**.



The optimal conditions for enzyme immobilization involve using sponge with a size of 0.125 cm³ as the immobilization material. Prior to immobilization, the sponge should be soaked in a solution of 0.1 molar phosphate buffer at pH 7.0 for 24 h and dried. Following this, the sponge should be soaked in the enzyme solution for a duration of 48 h. Under these conditions, the maximum enzyme activity achieved is 277 U/g of immobilization material. The optimization of various parameters on lipase immobilization, Effect of

immobilization materials, pH, size of immobilization materials and incubation periods on lipase activity. Moreover, study ability for thermo-tolerant, toluene tolerant on relative activity and physical description of immobilization materials after incubated with toluene and incubated at 60 °C. The immobilization materials characteristics are suitable for enzyme immobilization are stable, no toxic, resistant to condensation, difficult to physically change, and cheap. These characteristics of immobilization material will help to improve the stability and activity of enzymes [7-10].

The porous immobilization material that effects to homogenized between enzyme and substrate and enhance the mass transfer [11]. Porous materials are widely used for enzyme immobilization, pore structure and large surface area is suitable for adsorption of enzyme on materials, that depend on size of material [8-10].

The effect of pH on physical adsorption. In a previous study, the optimum pH of α -amylase enzyme binding on CB hybrid via adsorption method was the pH range of 7.0 and increase pH to 7.5 - 8.0 the enzyme activity decreased in the binding process. The result indicated that enzyme was denatured in alkaline pH and the immobilization process, enzyme can be bound on the immobilization material with the optimum pH. The enzyme can be bound to the surface of material by positively changing the active site of the hybrid surface by the cation-exchange process. Enzyme adsorption on the positively changing material and negative charging on an enzyme molecule due to electrostatic attraction at a pH range of 7.0 - 8.0 [10, 12].

The effect of temperature on thermo-stability of immobilized enzymes. This study results similarly to the previous report, the effect of temperature for α -amylase enzyme stability at 70 °C. The relative activity of immobilized enzyme on sugilite BSF glass was higher than free enzyme 1.4-fold. The result indicated that the immobilization of enzyme on sugilite BSF glass that enhanced enzyme has higher stability than free enzyme [13]. In previous reports, immobilized lipase from *Yarrowia lipolytica* on octyl-agarose and octadecyl-sepabeads via adsorption method. The result shows yield of enzyme activity and enzyme stability is higher than free enzyme was 10-folds [14]. Immobilized lipase from *Candida rugosa* by adsorption on 3-hydroxybutyrate-co-hydroxyvalerate that showed residual activity 94 % after incubated at 50 °C for 4 h and reusable for 12 times [15]. Moreover, lipase produced by *Aspergillus niger* was immobilized on luffa discs by use the initial enzyme concentration was 1 mg/mL and incubation time for 12 h. The maximum enzyme activity was 84 % of enzyme solution. The use of DEAE cellulose with glutaraldehyde cross-linking proved to be highly effective for immobilization, providing great operational stability. The optimal temperature and thermal stability of the immobilized alpha-amylase shifted from 60 to 70 degrees Celsius, resulting in an increased half-life. However, the optimal pH remained unchanged, while the pH stability shifted from 6 to 7 [5]. These results indicated the immobilization technique improves the enzyme stability in high temperature and solvent and enzyme can reusable in many time that suitable in many applications [16].

Characterization of enzyme lipase for PLA-polymerization

Effect of temperature on lipase activity

In this investigation, the influence of temperature on the thermal tolerance of lipase enzymes was examined. The Lipase enzymes were subjected to 2 conditions: Enzyme immobilization and free enzyme. The enzymes were exposed to various temperatures (30 - 80 °C) for a duration of 1 h before being analyzed for enzyme activity.

The experimental results revealed that immobilized lipase enzymes in sponge exhibited thermal tolerance within the temperature range of 30 - 60 °C, with a relative activity ranging approximately from

140 to 190 %. As the temperature was raised to 70 - 80 °C, there was a slight reduction in relative activity observed. Conversely, free lipase enzymes exhibited thermal tolerance in the temperature range of 30 - 50 °C, with a relative activity ranging approximately from 120 to 145 %. When the temperature was increased to 60 °C, there was a 50 % reduction in relative activity. Furthermore, at temperatures of 70 and 80 °C, the enzymes were no longer viable, as depicted in **Figure 7**.

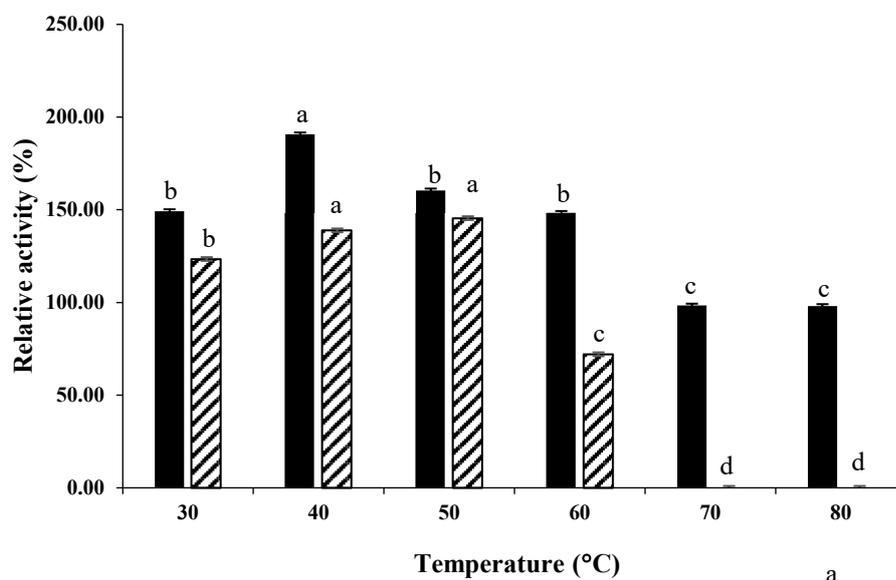


Figure 7 Effect of temperature on lipase activity. ■ ; immobilized lipase and ▨ ; crude enzyme

Efficiency of immobilized-lipase under PLA-polymerization condition

The study of the characteristics of immobilized lipase enzymes on sponge, it was observed that these enzymes exhibit resistance to high temperatures and can tolerate the presence of toluene. Therefore, the immobilized lipase enzymes on sponge were tested for their efficacy in the synthesis of poly (lactic acid) or PLA containing commercial exchangeable lactic acid as monomers at a concentration of 450 g/L. Toluene served as the solvent, and an additional 10 % of immobilized lipase enzymes were inoculated. The reactions were conducted at a temperature of 60 °C, and samples were taken hourly for a total of 8 h.

The experimental results revealed that the immobilized lipase enzymes maintained a relatively constant Relative activity of approximately 230 % within the reaction time of 0 - 5 h, with a slight reduction. the Relative activity values of the immobilized lipase on sponge was approximately 1.2 times higher than the free lipase, as shown in **Figure 8**.

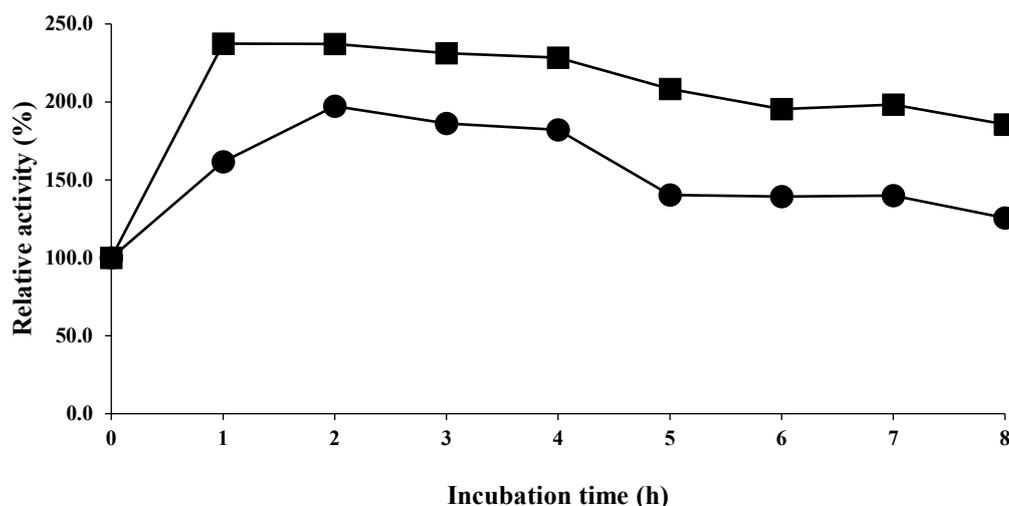


Figure 8 Efficiency of immobilized-lipase under PLA-polymerization condition. ■ ; immobilized lipase and ● ; Free-enzyme.

Consequently, the experimental findings suggest that immobilizing lipase enzymes on sponge can enhance their efficiency, leading to increased thermal stability compared to the use of free enzymes. This immobilization technique shows promise for future applications in the synthesis of poly (lactic acid) or PLA.

Application of immobilized-lipase on PLA-polymerization

The study of utilizing immobilized lipase enzymes produced from the strain *Streptomyces* sp. A3301 for the synthesis of poly (lactic acid), it was determined that the polymer could be successfully generated. The resulting polymer exhibited a molecular weight of $5,333 \pm 0.02$ Da and had a molecular weight per repeating unit of $(C_3H_4O_2)_n$, equivalent to 72 Da. Therefore, the degree of polymerization in the polymer was calculated to be 74, which was higher than the poly (lactic acid) synthesized using free enzymes. In that case, the polymer exhibited a molecular weight of only 525 Da, with a degree of polymerization of 7 [4].

Consequently, the utilization of immobilized lipase enzymes on sponge allowed for the synthesis of poly (lactic acid) with a higher degree of polymerization compared to using free enzymes. The degree of polymerization was increased up to 10.5 times, and the reactions were conducted at a temperature of 60 °C under continuous stirring in an air atmosphere with the addition of nitrogen gas. The reactions were carried out for a duration of 8 h. Enzyme lipase applications in PLA-polymerization, lipase was used in Poly (DL- lactic acid) PDLLA polymerization process as catalyst in esterification reaction and inhibition of enzyme activity. Generally, crude enzymes cannot be used in liquid form because water in the crude enzyme affects the esterification reaction. The lipase has been functioning in monophasic and low-water-activity systems [17-21]. Therefore, the water in immobilized-enzyme was removed by freeze drying technology before adding in the PLA-polymerization reaction and continuous homogenization to improve interaction between the substrate and enzyme [4,22,23] In our previous study, PDLLA polymerization by using crude lipase from *Streptomyces* sp. A3301 as biocatalyst. The result showed the highest molecular weight of PDLLA was 577 Da or a degree of polymerization of approximately 8 under optimized

conditions of 10 % dried lipase incubated at 60 °C for 8 h under a nitrogen atmosphere [4]. The result indicated that crude lipase from *Streptomyces sp.* A3301 has the ability to polymerization of PDLLA but yield of product was rather low. Therefore, in this study improve the PDLLA polymerization by using immobilized enzyme because the immobilized enzyme enhances the enzyme stability. So, immobilized enzymes can constantly be active in the reaction with toluene and temperature at 60 °C and impact to increase the molecular weight of PDLLA. The resulting polymer exhibited a molecular weight of $5,333 \pm 0.02$ Da and the degree of polymerization in the polymer was calculated to be 74, which was higher than the poly (lactic acid) synthesized using free enzymes. In addition, lipase B from *Candida antarctica* (CALB) was used in flavor ester production, specifically of butyl acetate by using both of free enzyme and immobilized enzyme, the immobilization improves the enzyme stability and activity, reduce inhibition of enzyme [16,22-24]. In previous studies, Novozym 435, the enzyme immobilized by poly (methyl methacrylate) crosslinked with divinylbenzene [25]. was used for butyl acetate synthesis, obtaining high conversion (over 90 %) in relatively short reaction times (2.5 h) [16]. PLA polymerization by using lipase at the optimal condition at 65 °C for 96 h by using hexane as solvent [26]. Chuensangjun reported that PLA with an Mn in the range of 2,600 - 4,500 was investigated by using the commercial lipase Lipozyme TLIM at an optimum temperature of 50 °C for 5 h in toluene under atmosphere with nitrogen [27]. The re-polymerization of PDLLA from a degradation product (lactic acid) by using the commercial lipase Lipozyme TL IM as a catalyst under a nitrogen atmosphere for 6 h. A re-polymerized PLA oligomer with a molecular weight of 378 Da or a degree of polymerization of approximately 5 was generated [28]. The study examined key factors influencing the production of Lactic Acid (LA). The Cellulosic Agricultural Biomass Hydrolysate (CABH) primarily contained glucose and xylose, with concentrations around 40 g/L and 20 g/L respectively. The process was carried out at 30 °C and 100 rpm, using cultures in the final growth phase (24 h for LAB07 and 20 h for LAB14) and urea as a nitrogen source. This approach boosted LA production for both LAB07 and LAB14 strains, achieving a yield of 24.3 g/L. The LA produced was then utilized to synthesize Polylactic Acid (PLA) via an enzymatic route using Lipase B from *Candida antarctica* (CALB) [29].

Conclusions

An optimal condition for enzyme immobilization involves utilizing sponge with a size of 0.125 cm^3 as the best immobilization material. Prior to immobilizing the enzyme on the sponge, the sponge should be soaked in a solution of 0.1 M phosphate buffer pH 7.0 at 4 °C for a duration of 24 h. Subsequently, the sponge is immersed in the enzyme solution for 48 h. Under these conditions, the enzyme exhibited the highest activity, reaching 277 U/g of immobilization material. Subsequently, the characteristics of the immobilized lipase enzymes on sponge, it was found that they could withstand temperatures ranging from 30 to 60 °C, with a relative activity of approximately 140 - 190 %. However, when the temperature was increased to 70 - 80 °C, there was a slight reduction in relative activity. In terms of synthesizing poly (lactic acid), the study found that immobilized lipase enzymes exhibited a relatively constant relative activity of approximately 230 % within the first 5 h, with a minor reduction thereafter. From these experimental findings, it can be concluded that the method of immobilizing enzymes using sponge can enhance the enzymes tolerance to high temperatures and toluene solvents. Additionally, when applied in the synthesis of poly (lactic acid), the immobilized lipase enzymes produced from the *Streptomyces sp.* A3301 strain demonstrated the ability to produce polymers with a molecular weight of $5,333 \pm 0.02$ Da and the degree of polymerization in the polymer was 74. This was considerably higher than the poly (lactic acid) synthesized using crude enzymes, with a 10.5-fold increase [4], all conducted at a temperature of

60 °C with continuous stirring under ambient conditions by nitrogen gas supplementation for a reaction time of 8 h. Consequently, this research suggests that the enzyme immobilization technique, particularly on sponge, can significantly enhance thermal and solvent stability, thereby offering potential for improving the synthesis of poly (lactic acid). This knowledge can also be further developed and applied to future research endeavors.

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