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Immobilization of poly(L-lactide)-degrading enzyme from *Laceyella sacchari* LP175: characterization and evaluation for hydrolysis of poly(L-lactide) polymer

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Abstract

Poly(L-lactide) (PLLA)-degrading enzyme from the thermophilic filamentous bacterium, *Laceyella sacchari* LP175, was entrapped in calcium alginate beads and characterized using emulsified PLLA as a substrate. The 1.0% sodium alginate appeared to be the most effective for the immobilization, with 60% actual immobilization efficiency and 80% theoretical immobilization efficiency. Immobilized enzyme represented emulsified PLLA hydrolyzing activity similar to free enzyme. The immobilized enzyme showed an operational stability up to five times that of the free enzyme, indicating that it is a suitable choice for applications to hydrolysis the PLLA polymer due to reduced enzyme preparation costs. The optimum temperature and thermal stability of the immobilized PLLA-degrading enzyme were shifted from 60 to 65 ^{o}C and 55 to 70 ^{o}C, respectively, while the pH optima and stability remained unaltered. The immobilized enzyme showed a higher stability at 60 ^{o}C for up to 12 h and improved the lactic acid tolerance ability up to 10% (v/v) as compared to the free enzyme which could help avoid lactic acid feedback inhibition during hydrolysis. Hence, the PLLA-

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degrading enzyme from *L. sacchari* LP175 was more stable after immobilization and represented a highly appropriate choice for the recycling process of emulsified PLLA polymer.

Keywords: Poly(L-lactide)-degrading enzyme, *Laceyella sacchari* LP175, Immobilization, Biodegradation

1. Introduction

PLLA-degrading enzyme comprises a group of enzymes that can degrade PLLA polymer to the short chain molecules such as dimers and monomers of L-lactic acid for industrial recycling applications [1]. Biological degradation of PLLA polymer by PLLA-degrading enzyme has received significant attention because it plays an important role in resolving the global warming problem [2]. Several PLLA-degrading microorganisms have been reported such as thermophilic bacteria, actinomycetes, and fungi [3, 4] Compared to actinomycetes, the bacterial degradation of PLLA has rarely been reported in the literature. Recently, the serine protease produced by the thermophilic filamentous bacterium, *L. sacchari* LP175, was reported as having PLLA depolymerase potential and being applicable to PLLA biodegradation, which was a focus in this work [1]. The production of PLLA-degrading enzyme from *L. sacchari* LP175 was investigated in a 3.0 L airlift bioreactor, with the crude enzyme being able to hydrolyze PLLA powder at 50 ^{o}C within 72 h [1].

To reduce global environmental problems, recycling of PLLA polymer by enzymatic degradation has received significant attention. Youngpreda *et al*. [2] successfully re-polymerized the PLLA hydrolysis product to a PLLA oligomer. The main problem for degradation of PLLA polymers is feedback inhibition from the accumulation of lactic acid during the hydrolysis. This issue was confirmed by the study of Lomthong *et al*. [1] who reported that the accumulation of 0.8 g/L lactic acid in the hydrolysis reaction inhibited 50% of PLLA-degrading enzyme activity. To avoid this problem, the immobilization of enzyme is a good choice for the recycling process. Immobilization is a method to enhance the stability and reusability of enzyme [5].

Immobilized enzyme by entrapment method is the process to entrap the enzyme with the matrix of polymer such as calcium alginate which easy for preparation, low cost and good biocompatibility [6]. This process will help to reduce the cost of enzyme preparation, increase the resistance to changes such as pH and temperature, improve product recovery and does not contaminate the final product [6, 7]. In addition, the immobilization of PLLA degrading enzyme not only increases the usable round but also avoids contamination of proteins (enzyme and media) and metals from crude enzyme to the hydrolysis product for the re-polymerization process [8, 9].

This study, therefore, aims to immobilize PLLA-degrading enzyme produced from the thermophilic filamentous bacterium, *L. sacchari* LP175, using an entrapment in calcium alginate method. The characteristics of the immobilized

enzyme in terms of hydrolysis of emulsified PLLA polymer are also investigated.

2. Materials and Methods

2.1 Microorganism and inoculum preparation

PLLA-degrading bacterium, *L. sacchari* LP175 was kept at the Department of Microbiology, Faculty of Science, Kasetsart University. The culture grown on the nutrient agar was inoculated to 50 mL of nutrient broth (3 g/L beef extract and 5 g/L peptone). The culture was cultivated at pH 7.0, 50 °C and 150 rpm for 12 h. The cell pellets after centrifuged (10,000 rpm) at 4 ^{o}C for 10 min were dispersed in sterile 0.85% (w/v) NaCl solution which adjusted the optical density of 0.5 at a wavelength of 600 nm. 10% (v/v) inoculum was used for cultivation.

2.2 PLLA-degrading enzyme production

PLLA-degrading enzyme produced from *L. sacchari* LP175 was performed in a 3.0 L airlift fermenter using a 2.0 L working volume of the optimized medium: 4.64 g/L cassava chips, 1.53 g/L soybean meal, and 0.31 g/L PLLA powder. 10% (v/v) inoculum of *L. sacchari* LP175 was added to the sterilized medium. The fermentation was operated at 50 ^{o}C, pH 7.0 at an aeration rate of 0.5 vvm for 24 h [1]. The obtained culture broth was centrifuged (10,000 rpm) at 4 ^{o}C for 10 min. The clear supernatant was used for enzyme immobilization.

2.3 PLLA-degrading activity assay

PLLA-degrading activity was determined by measuring the decreasing of PLLA emulsion turbidity at 60 ^{o}C, pH 9.0 for 30 min with the obtained enzyme, following the method described by Sukkhum *et al*. [10] One unit of PLLA-degrading activity was defined as a 1 unit decrease in optical density at 630 nm per min under the assay conditions described.

2.4 Immobilization of PLLA-degrading enzyme

The 5.0 mL of PLLA-degrading enzyme was added into 10 mL of the sodium alginate solution (Laboratory grade, Sigma, USA) (1.0–3.0% (w/v)). The enzyme-alginate mixture was added dropwise into calcium chloride (0.2 M) solution with continuous shaking at 4 °C. As soon as the drop of beads thus formed, the mixture was stirred slowly at 4 ^{o}C overnight. The unbound enzyme was washed with 0.1 M Tris-HCl buffer of pH 9.0 and immobilized enzymes preserved at 4 ^{o}C.

2.5 Assay of immobilized enzyme

For the enzyme assay, 0.25 g of calcium-alginate entrapped enzyme was added into 2.25 mL of 0.1% PLLA emulsion in 100 mM of Tris buffer (pH 9.0). The reaction was incubated at 60 ^{o}C for 30 min at 110 rpm [5]. One unit of PLLA-degrading activity was defined as a one unit decrease in optical density at 630 nm per min, as described above. The theoretical immobilization efficiency and practical immobilization efficiency was calculated using the following expression:

Theoretical immobilization efficiency (%)

$$= \frac{(A - B)}{A} \times 100$$

Actual immobilization efficiency (%) $= \frac{C \times 100}{A}$

where A is the total activity of the free enzyme; B is the total activity of the unbound enzyme and C is the total activity of the immobilized enzyme.

2.6 Operational stability of immobilized enzyme

The immobilized enzymes were incubated with 2.25 mL of 0.1% PLLA emulsion in 100 mM of Tris buffer (pH 9.0) at 60 ^{o}C for 15 min. The immobilized enzyme was separated and washed with 100 mM of Tris buffer (pH 9.0) used for the next reaction as described above. The process was repeated until the measurable enzyme activity was not detected [5].

2.7 Characterization of free and immobilized enzyme

The selected immobilized enzyme with high efficiency and operational stability was used for characterization with respect to the effect of temperature and pH.

2.7.1 Temperature and pH optima

To determine the temperature profile, the immobilized and free enzymes were incubated in the reaction at 30 to 80 ^{o}C which maintained the pH of the reaction at 9.0. Similarly, the effect of pH on the immobilized and free enzyme was studied over a pH range of 5–11. The enzyme assay was carried out with the substrate prepared in buffers with varying the pH. The reaction mixtures were incubated at the optimum temperature as described above.

2.7.2 Temperature and pH stability

The stability of the free and immobilized enzyme was monitored at various temperatures in the range of 30–80 ^{o}C for 1 h. Aliquots were withdrawn at different time intervals and PLLA-degrading activity determined. In a similar manner, the pH stability of the enzyme was monitored at pH 3.5–11.0 for 24 h and optimum stable temperature. The residual enzyme activity was then determined [5, 11]

2.7.3 Thermostability

The thermostability of enzyme was investigated by incubating the immobilized enzyme at different temperatures 4, 50, 60 and 70 ^{o}C for 12 h. The residual activities of immobilized enzyme at interval times were assayed under the optimal conditions which compared to the free enzyme at the same conditions.

2.8 Effect of lactic acid concentrations on free and immobilized enzymes tolerance

The immobilized enzyme was assayed under the optimal conditions with different concentrations of lactic acid (0, 1.0, 5.0 and 10.0% (v/v). The residual activities of immobilized enzyme were calculated as relative activity and compared with the free enzyme.

3. Results and Discussion

3.1 Immobilization of enzyme by entrapment method

Immobilization of PLLA-degrading enzyme for hydrolysis of emulsified PLLA solution is an alternative choice for recycling of PLLA polymers. This approach not only improves recovery and reusability of enzyme but also avoids contamination of enzyme and other compounds in the product solution. Compared to free enzymes in solution, immobilized enzymes are more robust and more resistant to environmental changes [6, 12]. The alginate beads' immobilization of PLLA-degrading enzyme from *L. sacchari* LP175 is shown in fig. 1A. The immobilized enzyme in calcium alginate could hydrolyze emulsified PLLA as shown in fig 1B (which illustrates clear and less particles of PLLA in the supernatant). This result confirms that the immobilized enzyme in calcium alginate could be applied for hydrolysis of emulsified PLLA solution to the lactic acid monomers.

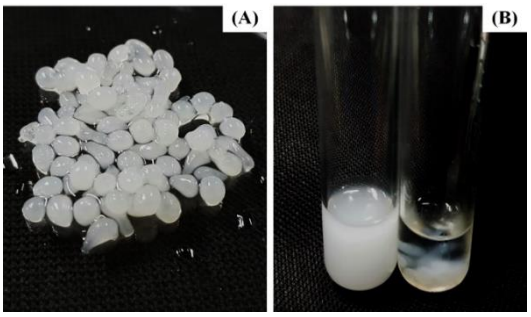

Figure 1 Alginate beads immobilization of PLLA-degrading enzyme from *L. sacchari* LP175 (A) and emulsified PLLA before and after hydrolyzed by immobilized enzyme (B).

The immobilization efficiency is depicted in Table 1. The immobilized PLLA-degrading enzyme produced by *L. sacchari* LP175 showed emulsified PLLA hydrolyzing activity similar to free enzyme and it was stable. The PLLA-degrading enzyme was entrapped in different concentrations of sodium alginate (1.0–3.0% (w/v)) which showed 60.28, 45.35, and 27.75% immobilization yield, respectively. The theoretical immobilization efficiency of immobilized enzymes were 80.4, 80 and 81.4 %, respectively. With increasing concentrations of sodium alginate, the immobilization efficiency of the enzyme was reduced. Singh [13] reported that at high alginate concentration, causes the substrate diffusion limitation which decreased yield and immobilization efficiency. Noreen *et al.* [14] reported that sodium alginate and $CaCl_2$ concentration are major parameters for enzyme entrapment in gel brads due to gel formation by cross-linkage between alginate and Ca^{2+} ion. In this study, the immobilized enzyme of all concentrations of sodium alginate were further investigated with respect to operational stability.

Table 1 Immobilization efficiency of PLLA-degrading enzyme using entrapment technique

Sodium alginate (%w/v)	Actual immobilization efficiency (%)	Theoretical immobilization efficiency (%)
1.0%	60.28 ± 1.51	80.41 ± 0.7
2.0%	45.35 ± 1.54	80.02 ± 1.41
3.0%	27.75 ± 1.65	81.36 ± 1.06

3.2 Operational stability of immobilized enzyme

The operational stability of an immobilized enzyme is one of the important parameters in the biotransformation processes. Immobilization could enhance the operational stability by making the process less costly and more sustainable under operational conditions [15]. The PLLA-degrading enzyme which immobilized on the 1.0% alginate was significantly stable and retained almost 30% of its initial activity for five cycles, as shown in Table 2. While the residual activities of 2.0% and 3.0% sodium alginate were less than 20% of its initial activity at four cycles (Table 2). The continuous loss in enzyme activity during successive cycles may be due to the leakage of the enzyme from the matrix, denaturation of the enzyme, or lactic acid inhibition. Anwar *et al*. [16] also used calcium alginate for immobilization of protease from *Bacillus subtilis* KIBGE-HAS, and found that immobilized enzyme can be reused up to three times. PLLA-degrading enzyme significantly entrapped in 1.0% sodium alginate showed high immobilization yield and operational stability. This observation suggested that the immobilized enzyme may be suitable for hydrolyze PLLA polymers as a clean technology and a sustainable process.

Table 2 Operational stability of immobilized enzyme entrapped with different concentrations of sodium alginate

Sodium alginate (%w/v)	% Residual activities of the immobilized enzyme					
	Number of cycles					
	1	2	3	4	5	6
1.0%	100	83.45	60.39	44.33	30.32	15.16
2.0%	100	67.11	34.89	16.51	10.41	1.65
3.0%	100	46.11	25.61	14.04	11.15	0.67

3.3 Characterization of free and immobilized enzyme

The effects of temperature on the stability of the free and immobilized enzymes were studied at temperatures of 30–80 ^{o}C. The free enzyme had high stability at 30–50 ^{o}C; however, on immobilization, the stability of the enzyme improved up to 65 ^{o}C (fig. 2).

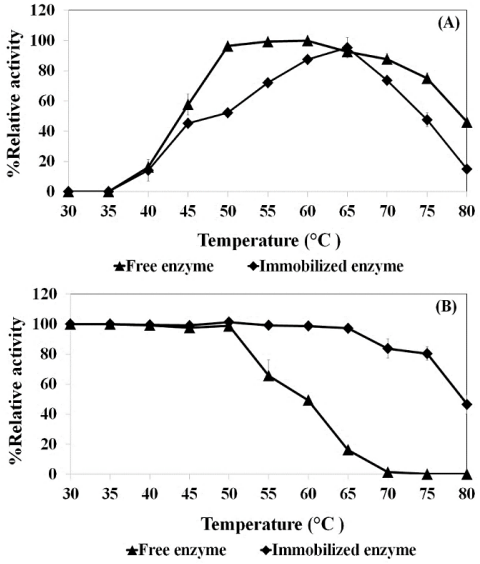

Figure 2 Effect of temperature on activity and stability of free and immobilized enzyme. (A) Optimum temperature of free and immobilized enzyme, (B) Stability of free and immobilized enzyme.

The improved thermal stability appears to be due to the binding of enzyme with the matrix. The matrix generally has a protective effect on the enzyme at elevated temperatures [5, 17]. The effect of pH on activity and stability of enzyme with and without immobilization was shown in fig. 3. The free enzyme showed maximum stability at pH 9.0, which was the same as for the immobilized enzyme according to this enzyme was characterized as a serine protease which high activity at high value of pH [11]. The stability of a free enzyme is principally determined by its intrinsic structure, whereas the stability of an immobilized enzyme is related to many factors such as the nature of its interaction with the carrier, binding position, and number of bonds [18, 19].

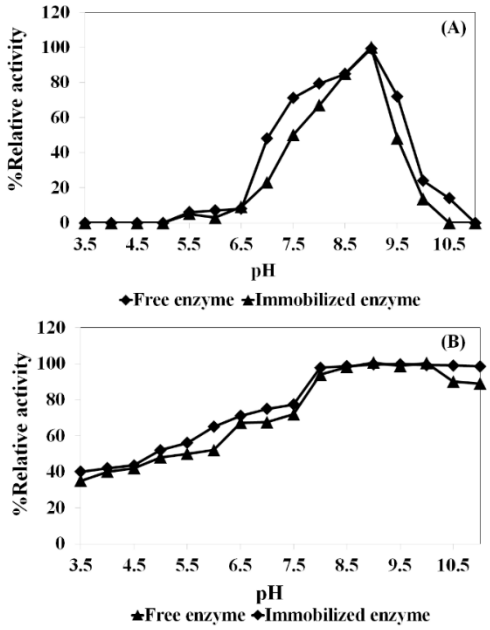

Figure 3 Effect of pH on activity and stability of free and immobilized enzyme. (A) Optimum pH of free and immobilized enzyme, (B) pH stability of free and immobilized enzyme.

3.4 Thermostability

To improve free enzyme thermostability, cofactors, metals, and other additives which play an important role in enzyme stability are typically used [20]. But the addition of other additives to the reaction causes contamination to the plastic that is difficult to purify before re-polymerization. In this

study, the improvement of thermostability by entrapment immobilization was thus investigated. The results for thermostability of free and immobilized enzyme are shown in fig. 4. At 4 ^{o}C, the residual activity both of free and immobilized enzyme remained high (100%) for 12 h. At 50 ^{o}C, the free enzyme slightly decreased after being incubated for 12 h (fig. 4A). But at 60 and 70 ^{o}C, the residual activity of free enzyme decreased to 70 and 40%, respectively. The immobilized enzyme showed high stability at 4, 50 and 60 ^{o}C with more than 95% remaining activity after being incubated for 12 h (fig. 4B), illustrating its higher stability than free enzyme under the same incubation conditions. This result indicated the improvement of thermostability of the PLLA-degrading enzyme produced by *L. sacchari* LP175 by the immobilization process.

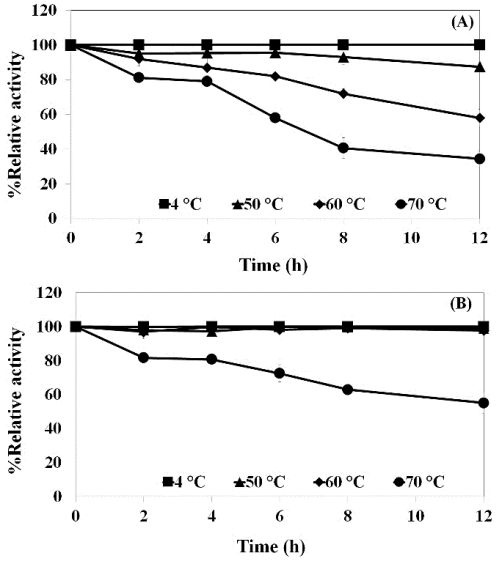

Figure 4 Storage stability of free and immobilized enzyme at different temperature. (A) Free enzyme, (B) Immobilized enzyme.

3.5 Effect of Lactic acid concentrations on free and immobilized enzymes tolerance

In terms of PLLA polymer hydrolysis, lactic acid liberated during the hydrolysis is the main issue [21]. As reported by Lomthong *et al.* [1], the accumulation of 0.8 g/L lactic acid in the hydrolysis reaction inhibited 50% of PLLA-degrading enzyme activity. In this study, the immobilized enzyme improved lactic acid tolerance up to 10% (v/v) as compared to the free enzyme (fig. 5). This result could be explained by the protection of alginate that entraps enzyme inside the structure. The liberated lactic acid was not able to contact directly at the allosteric site of the enzyme, thus helping to avoid the feedback inhibition of lactic acid in the reaction during the hydrolysis.

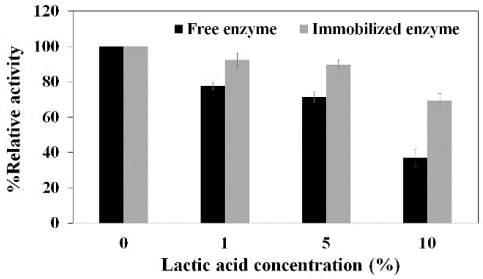

Figure 5 Effect of lactic acid concentrations on activity of free and immobilized enzyme.

4. Conclusions

The PLLA-degrading enzyme from the thermophilic filamentous bacterium, *L. sacchari* LP175, was immobilized in calcium alginate beads through an entrapment immobilization method,

which enhanced the optimum temperature. The immobilized enzyme also showed better stability and turnover operational stability and can be reused at least three times. In addition, the immobilized PLLA-degrading enzyme showed improved thermostability and lactic acid tolerance. This study illustrates the feasibility for application of immobilized enzyme in terms of recycling processes of emulsified PLLA polymer that becomes more stable after immobilization.

5. Acknowledgements

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