Electronic Journal of Biotechnology 30 (2017) 71–76

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

Poly(DL-lactide)-degrading enzyme production by immobilized *Actinomadura keratinilytica* strain T16-1 in a 5-L fermenter under various fermentation processes

Titiporn Panyachanakul [a], Vichien Kitpreechavanich [b], Shinji Tokuyama [c], Sukhumaporn Krajangsang [d,*]

[a] Department of Microbiology, Faculty of Science, Srinakharinwirot University, Watthana, Bangkok 10110, Thailand
[b] Department of Microbiology, Faculty of Science, Kasetsart University, Jatujak, Bangkok 10900, Thailand
[c] Department of Biological Chemistry, Faculty of Agriculture, Shizuoka University, Shizuoka, Japan
[d] Department of Microbiology, Faculty of Science, Srinakharinwirot University, Watthana, Bangkok 10110, Thailand

ARTICLE INFO

Article history:
Received 5 May 2017
Accepted 8 September 2017
Available online 21 September 2017

Keywords:
Actinomycete
Biodegradable polymer
Bioreactor
Cell immobilization
Depolymerase
Fermentation
Immobilization
Plastic wastes
Poly(DL-lactic acid)

ABSTRACT

Background: Poly(DL-lactic acid), or PDLLA, is a biodegradable polymer that can be hydrolyzed by various types of enzymes. The protease produced by *Actinomadura keratinilytica* strain T16-1 was previously reported to have PDLLA depolymerase activity. However, few studies have reported on PDLLA-degrading enzyme production by bacteria. Therefore, the aims of this study were to determine a suitable immobilization material for PDLLA-degrading enzyme production and optimize PDLLA-degrading enzyme production by using immobilized *A. keratinilytica* strain T16-1 under various fermentation process conditions in a stirrer fermenter.
Results: Among the tested immobilization materials, a scrub pad was the best immobilizer, giving an enzyme activity of 30.03 U/mL in a shake-flask scale. The maximum enzyme activity was obtained at aeration 0.25 vvm, agitation 170 rpm, 45°C, and 48 h of cultivation time. Under these conditions, a PDLLA-degrading enzyme production of 766.33 U/mL with 15.97 U/mL·h productivity was observed using batch fermentation in a 5-L stirrer fermenter. Increased enzyme activity and productivity were observed in repeated-batch (942.67 U/mL and 19.64 U/mL·h) and continuous fermentation (796.43 U/mL and 16.58 U/mL·h) at a dilution rate of 0.013/h. Scaled-up production of the enzyme in a 10-L stirrer bioreactor using the optimized conditions showed a maximum enzyme activity of 578.67 U/mL and a productivity of 12.06 U/mL·h.
Conclusions: This research successfully scaled-up the enzyme production to 5 and 10 L in a stirrer fermenter and is helpful for many applications of poly(lactic acid).

1. Introduction

Plastics provide social, economic, and environmental benefits, but their popularity has produced abundant plastic wastes. Disposing of plastic waste is a serious problem because plastics barely decompose in the environment. Biodegradable polymers such as poly(lactic acid) (PLA), poly-β-hydroxybutyrate (PHB), and polycaprolactone (PCL) have been developed as replacements for petrochemical plastic materials. PLA is an aliphatic polyester produced from lactic acid monomers. PLA can divide into several forms depending on the structure of lactic acid such as poly(LL-lactic acid) (PLLLA), poly(DL-lactic acid) (PDLLA), and poly(DD-lactic acid) (PDDLA). PLA has been used in many applications, e.g., food packaging, automotive interior parts, agricultural and geotextiles, furniture, apparel, and nonwovens, among others [1,2]. Disposal methods for PLA have been investigated, e.g., composting and recycling. To date, reports have demonstrated the ability of various microorganisms to degrade PLA, such as *Bacillus licheniformis, Bacillus* spp. *Geobacillus thermocatenulatus, Aneurinibacillus migulanus, Amycolatopsis thailandensis, Cladosporium, Purpureocillium, Laceyella sacchari, Thermoactinomyces vulgaris*, and *Actinomadura keratinilytica* [3,4,5,6,7,8,9,10]. However, the enzyme production by these reported strains and its scaling up have not been studied thus far. *A. keratinilytica* strain T16-1 showed the potential to produce a dominant activity of PDLLA-degrading enzyme. The basal medium composition that is used for the enzyme production was clarified and optimized. The maximum enzyme activity observed was 257 U/mL in an optimized basal medium containing PDLLA under batch fermentation in a 3-L airlift fermenter [11]. The main application of the PDLLA-degrading enzyme produced by strain T16-1 was biological recycling, as previously reported [11,12]. Currently, enzymes that degrade PLA, such as lipases and proteases, are used in many applications. In the plastic recycling field, these enzymes have been used for degradation and re-polymerization. Sukkhum et al.

* Corresponding author.
E-mail address: sukhumaporn@g.swu.ac.th (S. Krajangsang).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

https://doi.org/10.1016/j.ejbt.2017.09.001

[11] reported that almost 800 mg/L lactic acid and 500 mg/L oligomers were obtained after an 8-h degradation of 4000 mg/L PDLLA powder by strain T16-1. Youngpreda et al. [12] showed the biological recycling of PDLLA using a protease produced by *A. keratinilytica* strain T16-1 in a mild and clean process. In 2008, Lassalle and Ferreira [13] investigated the best conditions for the biological re-polymerization of PLA using lipase as a catalyst in hexane solvent. The results showed that PLA was obtained with 55% (w/v) under conditions of 65°C and 96-h reaction time. In 2012, Chuensangjun et al. [14] reported the optimized condition for PLA polymerization and demonstrated that low-molecular-weight PLA was derived using the commercial lipase Lipozyme TL IM at 50°C with 5-h incubation. A second strategy for the application of PLA-degrading enzyme was reported: the enzyme was mixed with a biopolymer film containing PLA to control the degradation when used in the agricultural field [15]. Moreover, a PLA-degrading enzyme might be used as a catalyst to improve the standard method for testing biodegradable polymers or co-polymers containing PLA [16].

To maximize enzyme production, the study of fermentation process development is highly important. In many reports, batch, fed batch, repeated-batch, and continuous fermentation were used to produce various types of fermentation products such as ethanol, lactic acid, lipid, and xylanase [17,18,19,20]. However, in each fermentation process, free cells were not suitable because of the possibility of substrate or product inhibition through direct contact between the cells and medium [21]; moreover, we lose time for new inoculum preparation and cleaning for each batch. To resolve these problems, a cell immobilization method was applied for the fermentation processes. Cell immobilization can help decrease the cost of fermentation and has many advantages such as achieving higher cell concentration, resulting in a higher fermentation rate, yield, and productivity and recycling cell utilization with reduced cost and time [22].

Therefore, the aim of this work focused on the comparison of fermentation processes using a 5-L stirrer fermenter to achieve the maximum PDLLA-degrading enzyme production with high yield and productivity.

2. Materials and methods

2.1. Microorganisms and inoculum preparation

The actinomycete *A. keratinilytica* strain T16-1 was isolated and identified by Sukkhum et al. [10] and kept at the NITE Biological Resource Center (NBRC), Japan, and Biotec Culture Collection (BCC), Thailand. Glycerol stock was prepared using 20% glycerol and stored at −20°C in refrigerator until use. Inoculum preparation was performed by streaking strain T16-1 onto ISP-2 slant (International Streptomyces Project-2 medium, containing 4 g/L yeast extract, 10 g/L malt extract, 4 g/L dextrose, and 20 g/L agar) and incubating at 45°C for 4 d. The strain was sub-cultured into ISP-2 broth and incubated at 45°C, 150 rpm, for 4 d. The cell mass was collected by filtration through Whatman No. 1 filter paper, washed twice, re-suspended in sterile distilled water, and used as the inoculum.

2.2. Substrate preparation and fermentation medium

PDLLA with a molecular weight (Mw) of 43,000 (80% L-lactic acid and 20% D-lactic acid, Toyobo, Japan) was used as the enzyme inducer in this study. PDLLA powder was prepared by adding 30-mL dichloromethane to a 0.3-g PDLLA pellet and mixing until the PDLLA pellet completely dissolved. To precipitate the PDLLA powder, 200 mL of methanol was added to the solution, which was then air-dried and used as the substrate. The composition of fermentation medium for the enzyme production was (w/v) 0.035% PDLLA powder, 0.238% gelatin, 0.4% $(NH_4)_2SO_4$, 0.4% K_2HPO_4, 0.2% KH_2PO_4, and 0.02% $MgSO_4.7H_2O$. Then, 10% (v/v) of seed culture was inoculated into the fermentation medium and incubated at 45°C for 4 d. The enzyme was collected by filtration through Whatman No. 1 filter paper, and the filtrate was further analyzed for enzyme activity.

2.3. Bacterial cell immobilization

The seed culture for cell immobilization was prepared as described above. Various carriers such as a luffa disc, sponge (Scotch Brite™ scrubbing sponge, 3M Thailand, Bangkok, Thailand), and scrub pad (Scotch Brite™ scrubbing pad, 3M Thailand, Bangkok, Thailand) were cut to a size of 1 cm^3. The scrub pad was made from aluminum oxide (non-fibrous), titanium dioxide, cured resin, and nylon fiber. Then, 10% inoculum was inoculated into sterile basal medium containing 0.1% (w/v) of each immobilizer. Calcium alginate immobilization was prepared separately. The seed culture was resuspended in 0.85% NaCl and sodium alginate at a final concentration of 10%. The cell suspension was dropped into 0.1 M $CaCl_2$ thrice, and the gel was collected, washed twice with sterile distilled water, and transferred to sterile basal medium. All flasks were incubated at 45°C for 7 d. Samples were taken at 24-h intervals and collected by filtration through Whatman No. 1 filter paper. The filtrate was further analyzed for enzyme activity.

2.4. Batch fermentation in a 5-L stirrer fermenter

The batch fermentation was conducted in a 5-L stirrer fermenter with a 4-L working volume (B. Braun Biotech Biostat B, Sartorius, Goettingen, Germany). The glass bioreactor was surrounded by a water jacket for temperature control. The dissolved oxygen (DO) and pH probes were positioned at the top of the fermenter. In total, 4 L of basal medium, 0.035% (w/v) PDLLA, and 0.1% (w/v) scrub pad were added into the fermenter and sterilized at 121°C for 30 min. Then, the mixture was inoculated with 10% (v/v) cell suspension, and fermentation was performed at various agitation speeds (100, 170, and 240 rpm) and aeration rates (0.25, 0.38, and 0.50) according to the experimental set up. The culture had a 6-h sampling interval for the PDLLA-degrading enzyme activity assay. The culture broth was obtained by filtration through Whatman No. 1 filter paper.

2.5. Repeated batch in a 5-L stirrer fermenter

The enzyme production by repeated-batch fermentation was performed according to the optimized conditions obtained for batch fermentation using a scrub pad as the immobilizer. The first cycle for repeated-batch fermentation was performed by culturing strain T16-1 in a 5-L stirrer fermenter with a 4-L working volume. Then, the mixture was inoculated with 10% (v/v) of the cell suspension, and the fermentation proceeded at 170 rpm, 0.25 vvm, and 45°C for 48 h. The culture broth was completely removed from the fermenter and resuspended in fresh sterile basal medium. The fermentation process was repeated until the enzyme production ability was decreased. The PDLLA-degrading enzyme activity assay was performed at a 6-h sampling interval. The culture broth was obtained by filtration through Whatman No. 1 filter paper.

2.6. Continuous process in a 5-L stirrer fermenter

The continuous process for PDLLA-degrading enzyme production was performed by culturing strain T16-1 in 4 L of basal medium containing 0.035% (w/v) PDLLA and 0.1% (w/v) scrub pad as immobilizer. A 10% (v/v) inoculum of the cell suspension was used, and the fermentation was controlled at 170 rpm, 0.25 vvm, and 45°C. The strain was grown until performance reached a steady state. The various dilution rates were 0.006, 0.013, 0.019, and 0.025/h. The culture was sampled at 6-h intervals for the PDLLA-degrading enzyme activity assay. The culture broth was obtained by filtration through Whatman No. 1 filter paper.

2.7. Batch fermentation in a 10-L stirrer fermenter

The PDLLA-degrading enzyme production was scaled-up to a 10-L stirrer fermenter with a 7-L working volume (B. Braun Biotech Biostat B, Sartorius, Goettingen, Germany). A 7-L volume of basal medium, 0.035% (w/v) PDLLA, and 0.1% (w/v) scrub pad were added into the fermenter and sterilized at 121°C for 30 min. A 10% (v/v) inoculum of cell suspension was used, and fermentation was conducted at an agitation speed of 170 rpm and aeration rate of 0.25 vvm. The culture was sampled at 6-h intervals for the PDLLA-degrading enzyme activity assay. The culture broth was obtained by filtration through Whatman No. 1 filter paper.

2.8. PDLLA-degrading enzyme assay

The PDLLA-degrading enzyme ability was determined by the decrease in emulsified PDLLA turbidity. For this purpose, 0.1% (w/v) PDLLA (80% L-lactic acid and 20% D-lactic acid with Mw of 43,000, Toyobo, Japan) was dissolved in dichloromethane, emulsified with 100-mM Tris–HCl buffer (pH 9.0) by an ultrasonic processor model VCX 500 (Sonic and Materials, Inc., Newtown, USA), and kept at room temperature to remove the solvent. A reaction mixture containing 2.25 mL of emulsified PDLLA and 0.25 mL of enzyme filtrate was incubated at 60°C for 30 min. The reaction was stopped by cooling on ice for 5 min. The decrease in turbidity was measured at 630 nm. One unit of PDLLA-degrading activity was defined as a 1-unit decrease in the optical density at 630 nm of emulsified PDLLA per milliliter under the assay conditions described above.

2.9. Scanning electron microscopy analysis

The cells immobilized on various carriers were cut into approximately 0.5-cm cubes, soaked into 2.5% glutaraldehyde prepared in 0.1 M phosphate buffer pH 7.2 at 4°C for 2 h, washed twice with the same buffer, and dehydrated by a series of ethanol concentrations for 10 min. The samples were dried and coated with gold before being observed under a scanning electron microscope (JEOL, JSM-6400, USA).

2.10. Calculation

The PDLLA-degrading enzyme production efficiency was calculated in terms of enzyme yield ($Y_{P/S}$) and enzyme productivity (Q_P).

$$Yeild\left(Y_{\frac{P}{S}}\right) = \frac{Enzyme\ activity\left(\frac{U}{ml}\right)}{Substrate\ concentration\left(\frac{g}{ml}\right)}$$

$$Enzyme\ productivity\ (Q_p) = \frac{Enzyme\ activity\left(\frac{U}{ml}\right)}{Fermentation\ time\ (h)}$$

2.11. Statistical analysis

The data were analyzed by one-way analysis of variance using the statistical program IBM SPSS Statistics for Windows, version 23.0 (IBM Corp., Armonk, N.Y. USA), and the LSD multiple comparison test was used for the separation of means. Statistical significance was applied at the $P < 0.05$ level.

3. Results and discussion

3.1. Immobilization of strain T16-1

In general, large-scale enzyme production requires several fermentation processes such as repeated-batch and continuous methods to maintain the yield and productivity. Thus, to increase the bacterial cell concentration in the process, immobilization and optimized conditions were studied. In this work, a luffa disc, sponge, scrub pad, and calcium alginate were used as cell immobilizers. Fig. 1 demonstrates the effect of various immobilizers on the enzyme production. The free cells, scrub pad, luffa disc, sponge, and calcium alginate showed enzyme activity levels of 31.17, 30.03, 25.83, 21.17, and 29.31 U/mL and productivity of 0.65, 0.63, 0.36, 0.18, and 0.31 U/mL·h, respectively, after 2 d of cultivation. Among these supports, the results of free cells and scrub pad were not significantly different. It can be concluded that the entrapment technique (calcium alginate immobilization) was not suitable for the cell immobilization of *A. keratinilytica* strain T16-1. In contrast, the attachment technique, especially using scrub pad immobilization, improved enzyme production. The immobilization efficiency of calcium alginate decreased because the substrate could not penetrate into the small pores of the beads. According to many reports, various attachment techniques have been successfully developed for cell and enzyme immobilization, e.g., stainless steel sponge, corn cob, alginate bead, and sweet sorghum stalk [19,23,24]. Hence, the scrub pad was selected for PDLLA-degrading enzyme production. The scanning electron micrograph of the immobilized strain T16-1 revealed that after cultivation for 48 h, the bacterial cells grew on the surface and inside the pores of the scrub pad (Fig. 2). The small mycelia of strain T16-1 were spread inside and attached well to the scrub pad. It was reported that cell attachment on a supporting surface consisted of non-covalent interactions such as van der Waals, ionic, electrostatic, and Lewis acid–base interactions and Brownian motion forces [25].

3.2. Batch fermentation for PDLLA-degrading enzyme production using a 5-L stirrer fermenter

From the results of cell immobilization, a scrub pad was used as the cell carrier to study enzyme production on a larger scale. The enzyme was produced by *A. keratinilytica* strain T16-1 using batch fermentation in a 5-L stirrer fermenter. Among various agitation speeds (100, 170, and 240 rpm), the highest enzyme production of 481.94 U/mL, productivity of 10.04 U/mL·h, and yield of 344.24 U/g substrate was achieved at 170 rpm and 0.38 vvm aeration rate

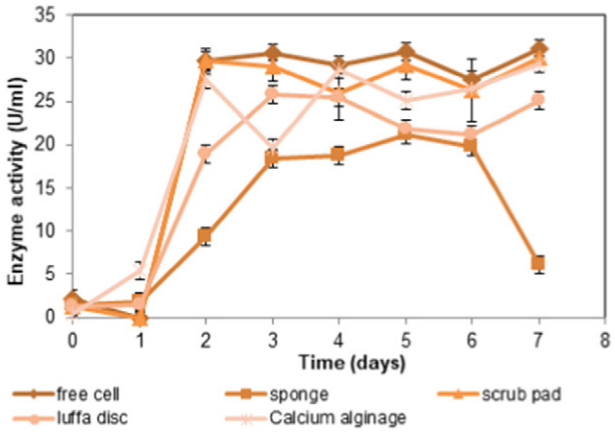

Fig. 1. Effect of various immobilizers on PDLLA-degrading enzyme production by *A. keratinilytica* strain T16-1 in 250-mL Erlenmeyer flasks, incubated at 150 rpm, 45°C, for 7 d. Closed diamond, free cell; closed square, sponge; closed triangles, scrub pad; ×, Calcium alginate; and closed circles, luffa disc.

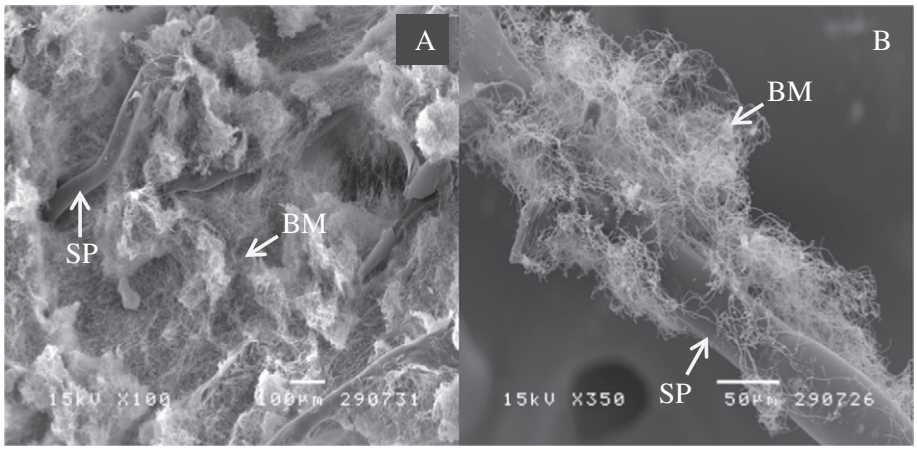

Fig. 2. Scanning electron micrograph of immobilized strain T16-1 on scrub pad after 48-h cultivation: (A) magnification of 100× and (B) magnification of 350×. SP: Scrub pad and BM: Bacterial mycelium.

(Fig. 3a). These results indicated that increasing the agitation speed could decrease the enzyme production because of the shear force of the disc turbine on mycelium growth. Fig. 3b demonstrates the effect of aeration rate (0.25, 0.38, and 0.5 vvm) on the PDLLA-degrading enzyme production by *A. keratinilytica* strain T16-1. The maximum enzyme activity, 766.33 U/mL, was obtained at 0.25 vvm with an agitation speed of 170 rpm under batch fermentation. A productivity (Q_p) of 15.97 U/mL·h and enzymatic yield ($Y_{P/S}$) of 547.38 U/g substrate were achieved under optimized conditions. It has been reported that enzymes are susceptible to mechanical force, which may disturb the elaborate shape of complex molecules severely enough to cause denaturation of the molecules [26]. High agitation speeds did not favor the enzymatic activity, probably because of the shear stress caused by the blade tips of the impeller, which increases with the revolution speed [27]. Stress conditions might negatively contribute to cell growth and enzyme stability [28].

3.3. Repeated-batch fermentation for PDLLA-degrading enzyme production using a 5-L stirrer fermenter

The best conditions for PDLLA-degrading enzyme production under batch fermentation were agitation speed of 170 rpm and aeration of 0.25 vvm. In addition, the enzyme was produced by repeated-batch fermentation because of the simplicity, convenience, and reduced time of operation and helped retain the cell mass when used for long periods of time or repeated fermentation. In this research, four successive cycles of enzyme production were performed (Fig. 4). In each cycle, high enzyme production was detected at 952, 1095.34, 996.67, and 726.67 U/mL. During repeated-batch fermentation, the maximum enzyme activity was achieved at the second cycle because the immobilized cells were activated after completing the first cycle of enzyme production. Afterward, the enzyme production slightly decreased when the immobilized cells were repeatedly used. The average production, productivity, and yield were 942.67 U/mL, 19.64 U/mL·h, and 673.34 U/g substrate, respectively (Table 1). These results demonstrated that the enzyme production was higher than the previously reported by Sukkhum et al. [11] by approximately

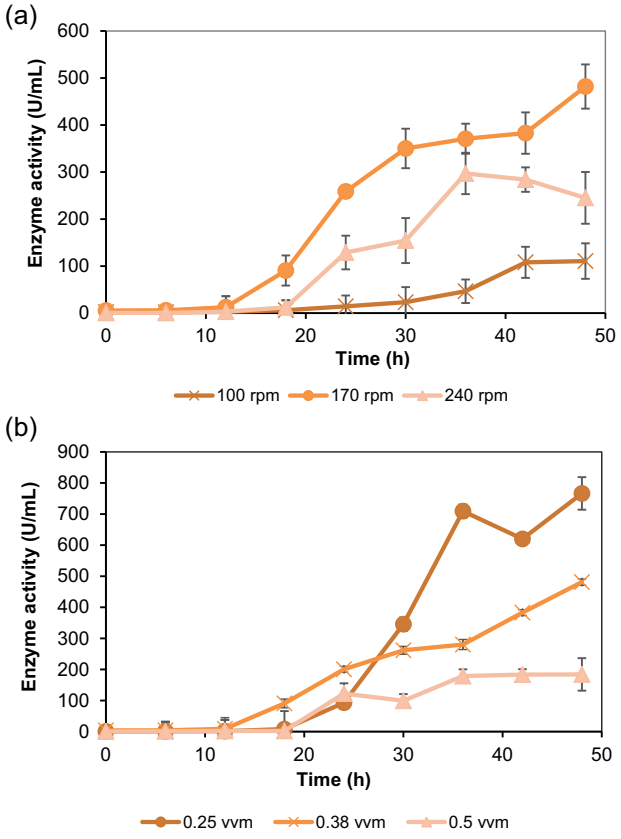

Fig. 3. Effect of agitation speed (a) closed square, 100 rpm; closed circles, 170 rpm; and closed triangles, 240 rpm and aeration rate (b) closed circles, 0.25; closed square, 0.38; and closed triangles, 0.5 vvm on PDLLA-degrading enzyme production by *A. keratinilytica* strain T16-1 in a 5-L stirrer fermenter under batch fermentation.

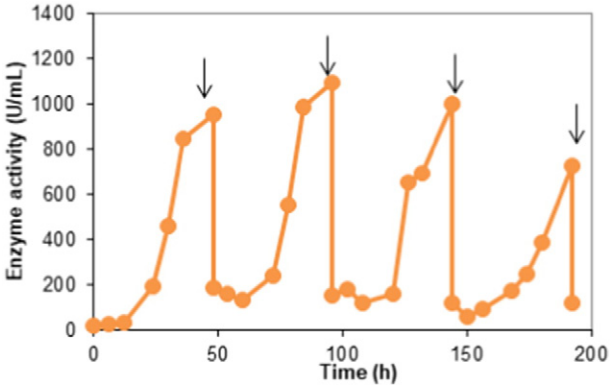

Fig. 4. PDLLA-degrading enzyme production by *A. keratinilytica* strain T16-1 in a 5-L stirrer fermenter under repeated-batch fermentation at aeration rate of 0.25 vvm, agitation speed of 170 rpm, and temperature of 45°C. The arrows indicate the start time of each cycle.

Table 1
Comparison of the fermentation parameters of PDLLA-degrading enzyme production by immobilized strain T16-1 under various fermentation processes in a 5-L stirrer fermenter.

Fermentation scale	Fermentation process	Parameters (mean ± SD)			Time (h)
		P (U/mL)	Q_p (U/mL·h)	$Y_{p/s}$ (U/g substrate)	
250 mL	Batch	30.03 ± 5^{d}	0.63 ± 0.1^{d}	150.15 ± 13^{d}	48
5 L	Batch	766.33 ± 32^{b}	15.97 ± 4^{b}	547.38 ± 24^{b}	48
	Repeated batch	942.67 ± 55^{a}	19.64 ± 2^{a}	673.34 ± 11^{a}	48
	Continuous	796 ± 43^{b}	16.58 ± 3^{b}	568.57 ± 14^{b}	48
10 L	Batch	578.67 ± 23^{c}	12.06 ± 1^{c}	165.33 ± 9^{c}	48

P, enzyme activity; Q_p, the enzyme productivity; and $Y_{p/s}$, the enzyme yield. The different letter Means the value at the same column are not significantly different at $P < 0.05$.

3.67-fold. Repeated-batch fermentation has been successfully used to produce important industrial products such as lactic acid, ethanol, lipid, and gamma-linolenic acid [17,19,20].

3.4. Continuous fermentation for PDLLA-degrading enzyme production using a 5-L stirrer fermenter

One of the major drawbacks in industrial applications of enzyme catalysis is the relatively high cost of the biocatalyst. Therefore, the production of high amounts of biocatalyst at a low cost is critical. Thus, it would be extremely interesting to develop technology for continuous production [29]. To evaluate a suitable fermentation process for PDLLA-degrading enzyme production, continuous fermentation was studied at various dilution rates of 0.006, 0.013, 0.019, and 0.025/h. After culturing for 48 h, the enzyme production reached a steady state, and new culture broth was continuously fed into a 5-L fermenter according to each dilution rate. The optimized condition for PDLLA-degrading enzyme production under continuous fermentation was a dilution rate of 0.013/h, an aeration rate of 0.25 vvm, an agitation speed of 170 rpm, and a controlled temperature of 45°C. The highest enzyme activity of 796 U/mL was obtained at a dilution rate of 0.013/h. The enzyme productivity obtained in continuous culture (16.58 U/mL·h) was lower than the production using repeated-batch fermentation. However, the benefit of continuous culture due to more efficient substrate use has been reported by many researchers [29,30]. At a high dilution rate, continuous fermentation (0.019 and 0.025/h) demonstrated enzyme production of 359.07 and 156.8 U/mL, respectively, which was lower than that in the optimized conditions. It could be concluded that a higher dilution rate decreased the enzyme activity, and the process reached the wash-out stage of fermentation. Similar behavior was reported for lipolytic enzyme production in continuous culture of *Thermus thermophilus* HB27. The lipolytic enzyme was approximately two-fold higher in continuous culture than in batch culture [29]. The xylanase productivity obtained in continuous culture of *Penicillium canescens* 10–10c (3.46 U/mL·h) was approximately 2.64 and 7.47 times higher than that in batch culture (1.31 and 0.46 U/mL·h) after 96 and 168 h of fermentation, respectively [31].

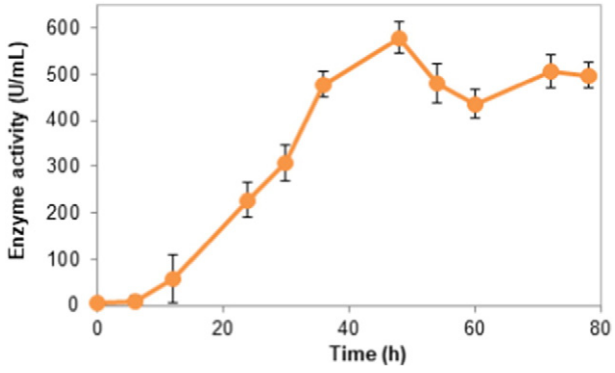

Fig. 5. PDLLA-degrading enzyme production by *A. keratinilytica* strain T16-1 in a 10-L stirrer fermenter under batch fermentation.The condition for the enzyme production was agitation speed of 170 rpm, aeration rate of 0.25 vvm, and controlled temperature of 45°C.

3.5. Scaling up for PDLLA-degrading enzyme production in a 10-L fermenter

The optimum conditions for PDLLA-degrading enzyme production by strain T16-1 under batch fermentation in the 5-L fermenter were scaled-up to a 10-L stirrer fermenter. The maximum enzyme activity of 578.67 U/mL was obtained after cultivation for 48 h at 45°C, with an agitation speed of 0.25 vvm and an aeration rate of 170 rpm (Fig. 5). The productivity and yield for the enzyme production were 12.6 U/mL·h and 236.19 U/g substrate, respectively. These results indicated that the enzyme production under batch fermentation in a 10-L fermenter was lower than that in a 5-L fermenter (Table 1) because the oxygen and mixing speed might not be suitable for scaling up to 10 L. Therefore, it was necessary to investigate good mixing of the culture broth as agitation produces dispersion of air in the culture medium, homogenizes the temperature and the pH, and improves the transference rate of nutrients.

4. Conclusion

The PDLLA-degrading enzyme production by strain T16-1 was successfully scaled-up to 5- and 10-L stirrer fermenters. The results show that a scrub pad is the best immobilizer for enzyme production with an activity of 30.03 U/mL in the shake-flask scale. The maximum enzyme activity is obtained at an aeration of 0.25 vvm, an agitation of 170 rpm, 45°C, and 48 h of cultivation time. Batch, repeated-batch, and continuous fermentation processes are used for the enzyme production. The maximum enzyme activity, 942.67 U/mL, is obtained after applying repeated-batch fermentation for PDLLA-degrading enzyme production in a 5-L fermenter. Scale-up of the enzyme in a 10-L stirrer bioreactor is demonstrated with an enzyme activity of 578.67 U/mL and productivity of 12.06 U/mL·h. These results can promote the scale-up of the production of the enzyme by the strain T16-1 and help in many applications of PLA.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Financial support

This work was supported by the National Research Council of Thailand (Grant number 2557-42).

References

[1] Xiao RZX, Zeng ZW, Zhou GL, et al. Recent advances in PEG–PLA block copolymer nanoparticles. Int J Nanomedicine 2010;5:1057–65. https://doi.org/10.2147/IJN.S14912.

[2] Jamshidian M, Tehrany EA, Imran M, et al. Poly-lactic acid: production, applications, nanocomposites, and release studies. Compr Rev Food Sci Food Saf 2010;9(5): 552–71. https://doi.org/10.1111/j.1541-4337.2010.00126.x.
[3] Arena M, Abbate C, Fukushima K, et al. Degradation of poly (lactic acid) and nanocomposites by *Bacillus licheniformis*. Environ Sci Pollut Res Int 2011;18(6):865–70. https://doi.org/10.1007/s11356-011-0443-2.
[4] Wu CS. Characterizing biodegradation of PLA and PLA-*g*-AA/starch films using a phosphate-solubilizing *Bacillus* species. Macromol Biosci 2008;8(6):560–7. https://doi.org/10.1002/mabi.200700181.
[5] Tomita K, Nakajima T, Kikuchi Y, et al. Degradation of poly(L-lactic acid) by a newly isolated thermophile. Polym Degrad Stab 2004;84(3):433–4386. https://doi.org/10.1016/j.polymdegradstab.2003.12.006.
[6] Hanphakphoom S, Maneewong N, Sukkhum S, et al. Characterization of poly (L-lactide)-degrading enzyme produced by thermophilic filamentous bacteria *Laceyella sacchari* LP175. J Gen Appl Microbiol 2014;60(1):13–22. https://doi.org/10.2323/jgam.60.13.
[7] Chaisu K, Charles AL, Guu YK, et al. Optimization of Poly Lactic Acid (PLA) plastic degradation by *Aneurinibacillus migulanus* using response surface methodology. International conference on biological and life sciences IPCBEE IACSIT press Singapore, Vol. 40. 2012. p. 22–7.
[8] Penkhrue W, Khanongnuch C, Masaki K, et al. Isolation and screening of biopolymer-degrading microorganisms from northern Thailand. World J Microbiol Biotechnol 2015;31(9):1431–42. https://doi.org/10.1007/s11274-015-1895-1.
[9] Prema S, Devi UM. Degradation of poly lactide plastic by mesophilic bacteria isolated from compost. Int J Res Pure Appl Microbiol 2013;3(4):121–6.
[10] Sukkhum S, Tokuyama S, Tamura T, et al. A novel poly(L-lactide) degrading actinomycetes isolated from Thai forest soils, phylogenic relationship and the enzyme characterization. J Gen Appl Microbiol 2009;55(9):459–67. https://doi.org/10.2323/jgam.55.459.
[11] Sukkhum S, Tokuyama S, Kitpreechavanich V. Poly(L-lactide)-degrading Enzyme production by *Actinomadura keratinilytica* T16-1 in 3L airlift bioreactor and its degradation ability for biological recycle. J Microbiol Biotechnol 2012;22(1):92–9. https://doi.org/10.4014/jmb.1105.05016.
[12] Youngpreda A, Panyachanakul T, Kitpreechavanich V, et al. Optimization of poly(DL-lactic acid) degradation and evaluation of biological re-polymerization. J Polym Environ 2016:1–9. https://doi.org/10.1007/s10924-016-0885-1.
[13] Lassalle VL, Ferreira ML. Lipase-catalyzed synthesis of polylactic acid: an overview of the experimental aspects. J Chem Technol Biotechnol 2008;83(11):1493–502. https://doi.org/10.1002/jctb.1994.
[14] Chuensangjun C, Pechyen C, Chisti Y, et al. Lipase-catalysed polymerization of lactic acid and the properties of the polymer. Adv Mat Res 2012;506:154–7. https://doi.org/10.4028/www.scientific.net/AMR.506.154.
[15] Chevallier A. Activity report 2015, CARBIOS reinvent polymers lifecycle: Thanaplast™. A first project and framework. [cited August, 3, 2017], p. 1-55. Available from Internet: https://carbios.fr/app/uploads/2016/04/1467122971_2015_Activity_Report_Carbios_2015.pdf.
[16] Lomthong T, Hanphakphoom S, Yoksan R, et al. Co-production of poly(L-lactide)-degrading enzyme and raw starch-degrading enzyme by *Laceyella sacchari* LP175 using agricultural products as substrate, and their efficiency on biodegradation of poly(L-lactide)/thermoplastic starch blend film. Int Biodeter Biodegr 2015;104: 401–10. https://doi.org/10.1016/j.ibiod.2015.07.011.
[17] Dashtia MG, Abdeshahian P. Batch culture and repeated-batch culture of *Cunninghamella bainieri* 2A1 for lipid production as a comparative study. Saudi J Biol Sci 2016;23(2):172–80. https://doi.org/10.1016/j.sjbs.2015.02.006.
[18] Dashti MG, Abdeshahian P, Yusoff WMW, et al. Repeated batch fermentation biotechnology for the biosynthesis of lipid and gamma-linolenic acid by *Cunninghamella bainieri* 2A1. Biomed Res Int 2014;2014:831783. https://doi.org/10.1155/2014/831783.
[19] Ariyajaroenwong P, Laopaiboon P, Laopaiboon L. Capability of sweet sorghum stalks as supporting materials for yeast immobilization to produce ethanol under various fermentation processes. J Taiwan Inst Chem Eng 2015;49:79–84. https://doi.org/10.1016/j.jtice.2014.11.016.
[20] Abdel-Rahman MA, Tashiro Y, Zendo T, et al. Improved lactic acid productivity by an open repeated batch fermentation system using *Enterococcus mundtii* QU 25. RSC Adv 2013;3:8437–45. https://doi.org/10.1039/C3RA00078H.
[21] Patkova J, Smogrovicova D, Domeny Z, et al. Very high gravity wort fermentation by immobilised yeast. Biotechnol Lett 2000;22(14):1173–7. https://doi.org/10.1023/A:1005689313775.
[22] Tzeng JW, Fan LS, Gan YR, et al. Ethanol fermentation using immobilized cells in a multi stage bed bioreactor. Biotechnol Bioeng 1991;38(10):1253–8. https://doi.org/10.1002/bit.260381019.
[23] López E, Deive FJ, Longo MA, et al. Lipolytic enzyme production by immobilized *Rhizopus oryzae*. Chem Eng Technol 2008;31(11):1555–60. https://doi.org/10.1002/ceat.200800289.
[24] Maghsoodi V, Kazemi A, Nahid P, et al. Alkaline protease production by immobilized cells using *B. licheniformis*. Sci Iran 2013;20:607–10.
[25] Bos R, Van Der Mei HC, Busscher HJ. Physico-chemistry of initial microbial adhesive interactions its mechanisms and methods for study. FEMS Microbiol Rev 1999; 23(2):179–230. https://doi.org/10.1111/j.1574-6976.1999.tb00396.x.
[26] Gupta R, Beg QK, Lorenz P. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 2002;59(1):15–32. https://doi.org/10.1007/s00253-002-0975-y.
[27] Pandey A, Soccol CR, Mitchell D. New developments in solid state fermentation: I- bioprocesses and products. Process Biochem 2000;35(10):1153–69. https://doi.org/10.1016/S0032-9592(00)00152-7.
[28] Thomas CR. Chemical engineering problems in biotechnology. In: Winkler MA, editor. Critical reports in applied chemistry. London: Elsevier Applied Science; 1990. p. 23–93.
[29] Dominguez A, Deive FJ, Pastrana L, et al. Thermostable lipolytic enzymes production in batch and continuous cultures of *Thermus thermophilus* HB27. Bioprocess Biosyst Eng 2009;33(3):347–54. https://doi.org/10.1007/s00449-009-0331-z.
[30] Racine FM, Saha BC. Production of mannitol by *Lactobacillus intermedius* NRRLB-3693 in fed-batch and continuous cell-recycle fermentations. Process Biochem 2007;42(12):1609–13. https://doi.org/10.1016/j.procbio.2007.09.001.
[31] Bakri Y, Akeed Y, Thonart P. Comparison between continuous and batch processing to produce xylanase by *Penicillium canescens* 10-10c. Braz J Chem Eng 2012;29(3): 441–7. https://doi.org/10.1590/S0104-66322012000300001.