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.