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Research article

Development of biodegradation process for Poly(DL-lactic acid) degradation by crude enzyme produced by *Actinomadura keratinilytica* strain T16-1

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ABSTRACT

Background: Plastic waste is a serious problem because it is difficult to degrade, thereby leading to global environment problems. Poly(lactic acid) (PLA) is a biodegradable aliphatic polyester derived from renewable resources, and it can be degraded by various enzymes produced by microorganisms. This study focused on the scale-up and evaluated the bioprocess of PLA degradation by a crude microbial enzyme produced by *Actinomadura keratinilytica* strain T16-1 in a 5 L stirred tank bioreactor.

Results: PLA degradation after 72 h in a 5 L bioreactor by using the enzyme of the strain T16-1 under controlled pH conditions resulted in lactic acid titers (mg/L) of 16,651 mg/L and a conversion efficiency of 89% at a controlled pH of 8.0. However, the PLA degradation process inadvertently produced lactic acid as a potential inhibitor, as shown in our experiments at various concentrations of lactic acid. Therefore, the dialysis method was performed to reduce the concentration of lactic acid. The experiment with a dialysis bag achieved PLA degradation by weight loss of 99.93%, whereas the one without dialysis achieved a degradation of less than approximately 14.75%. Therefore, the dialysis method was applied to degrade a commercial PLA material (tray) with a conversion efficiency of 32%, which was 6-fold more than that without dialysis.

Conclusions: This is the first report demonstrating the scale-up of PLA degradation in a 5 L bioreactor and evaluating a potential method for enhancing PLA degradation efficiency.

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1. Introduction

Currently, plastic wastes are a serious problem because they have a complex structure, which makes their degradation difficult. Plastic disposal methods such as incineration and landfilling are causes of global warming. Therefore, biodegradable plastics are of interest as replacements for petrochemical plastic materials. Biodegradable polymers and bioplastics are defined as any natural or synthetic substance engineered to interact with biological systems to direct medical treatment [1]. Bioplastics are defined as plastics based on renewable resources or plastics that are biodegradable or compostable [2]. Poly(lactic acid) or PLA is an aliphatic polyester produced from lactic acid, which is, in turn, produced by the action of microorganisms on agricultural residues as the substrate [3]. Presently, PLA is widely used in many commercial applications such as packaging, apparel, electrical appliances and electronics, and transportation [4]. The disposal methods for PLA should be determined, that is, their properties of biodegradation or biological recycling. To date, many reports have demonstrated the ability of various microorganisms to degrade PLA, such as *Bacillus brevis*, *Bacillus stearothermophilus, Bacillus licheniformis, Bacillus amyloliquefaciens*, *Geobacillus thermocatenulatus, Aneurinibacillus migulanus*, *Amycolatopsis thailandensis, Cladosporium, Purpureocillium, Laceyella sacchari, Thermoactinomyces vulgaris*,

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Actinomadura keratinilytica, *Nonomuraea* strains, *Thermoactinomyces vulgaris*, *Bordetella petrii* PLA-3, *Stenotrophomonas maltophilia* LB2–3, *Thermopolyspora flexuosa*, *Pseudonocardia* sp. RM423, *Stenotrophomonas pavanii,* and *Pseudomonas geniculata* [5,6,7,8,9,10,11,12,13,14]. In particular, *A. keratinilytica* strain T16-1 showed the potential to produce a PLA-degrading enzyme. The maximum enzyme activity was 766 U/mL and 943 U/mL in a 5 L stirred tank bioreactor by immobilized *A. keratinilytica* strain T16-1 on a scrub pad under batch and repeated batch conditions, respectively [15]. Microbial protease and lipase were reported to show the ability to degrade PLA. The first reported PLA-degrading enzyme was proteinase K from *Tritirachium album* [8]. Subsequently, there has been much research demonstrating that a PLA-degrading enzyme belonged to the protease group [8,11,12,16]. Protease can degrade PLA because of a recognized α-ester bond of PLA [16,17,18]. Lipase constitutes a significant group of esterases for enzymatic degradation of aliphatic polyesters. Lipase cleaves the ester bond randomly along the main chain of the polymer substrate. The application of both protease and lipase was focused on the PLA biological recycling process. Many reports showed the potential of the enzyme to degrade and synthesize PLA. In addition, the first report on the optimum condition for PLA degradation was reported by using a crude enzyme produced by *G. thermocatenulatus.* The enzyme could degrade PLA at Mw of 47,000 units at 60°C for 20 d, the degradation ability of which was measured by weight loss and using gel permeation chromatograms [7]. In 2004, Takahashi et al. [19] reported on PLA degradation by lipase. The results showed that poly(DL-lactic acid) or PLA of 84,000 units was degraded by lipase CA (Novozyme 435) to cyclic oligomer in organic solvents such as chloroform/hexane at 60°C and PLLA of 120,000 units was degraded at 100°C in the organic solvent o-xylene. Jarerat et al. [18] studied the comparison of PLA degradation by enzymes from *Amycolatopsis orientalis* and commercial proteinase K. The result showed 2000 mg/L PLA powder was degraded to 600 mg/L lactic acid by 20 mg/L enzyme at 40°C for 8 h, and the byproduct was measured for total organic carbon or TOC. In 2012, Sukkhum et al. [20] reported that PLA was degraded by protease from *A. keratinilytica* strain T16-1. The results showed 4000 mg/L PLA was degraded to 800 mg/L lactic acid and 500 mg/L other oligomers at 60°C for 8 h. Moreover, PLA powder was degraded by the crude enzyme from *A. keratinilytica* strain T16-1 under optimized conditions: the enzyme with an initial activity of 200 U/mL incubated at 60°C for 24 h released 6843 mg/L lactic acid with a conversion rate of 82%, which was similar to those of the commercial enzyme proteinase K (81%) [21].

In this research, we focused on the scale-up and bioprocessing of PLA degradation by a crude microbial enzyme. The aims of this work are to scale-up PLA degradation in a 5 L bioreactor and evaluate a potential method for the enhancement of PLA degradation efficiency.

2. Material and methods

2.1. Microorganisms and inoculum preparation

The actinomycete *A. keratinilytica* strain T16-1 was used as the producer of PLA-degrading enzyme in this study. The inoculum preparation was performed by shaking strain T16-1 in ISP-2 broth (International Streptomyces Project-2 medium containing 4 g/L yeast extract, 10 g/L malt extract, and 4 g/L dextrose) and incubating the inoculated broth at 45°C and 150 rpm for 4 d. The cell mass was collected by filtration through Whatman No. 1 filter paper, washed twice, resuspended in sterile distilled water, and used as the inoculum.

2.2. Substrate preparation and fermentation medium

Poly(DL-lactic acid) (PLA) with a molecular weight of 43,000 (80% L-lactic acid and 20% D-lactic acid, Toyobo, Japan) was used as the substrate in this experiment. PLA powder was prepared by adding 30 mL dichloromethane to a 0.3 g PLA pellet and mixing until the PLA pellet completely dissolved. To precipitate the PLA powder, 200 mL of methanol was added to the solution, which was then air-dried. The composition of the fermentation medium for the enzyme production was (*w*/*v*) 0.035% PLA 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 the seed culture was inoculated into the fermentation medium and incubated at 45°C for 4 d. The enzyme was collected by filtration through a filter paper (Whatman No. 1), and the filtrate was further analyzed for enzyme activity.

2.3. Optimization of PLA degradation in a 5 L bioreactor

The crude enzyme with an initial activity of 1000 U/mL was diluted to 200 U/mL by using 100 mM Tris–HCl at pH 9.0. The reaction mixture, containing 4 g/L PLA powder and 3.5 L of diluted enzyme, was added into a 5 L stirred tank bioreactor (B. Braun Biotech Biostat B, Sartorius, Goettingen, Germany). The reactor was controlled at 60°C with an agitation speed of 50 rpm and interval sampling every 6 h for 5 d. The sample was collected by centrifugation at 10,000 × *g* for 5 min at 4°C and subjected to analyze the lactic acid concentration by HPLC. The factors affecting PLA degradation was determined in a 5 L bioreactor. The effect of various agitation speeds on PLA degradation was studied at 50 and 100 rpm with an initial pH of 9.0 and controlled at 60°C. The effect of the pH values on PLA degradation was determined at pH 8.0, 9.0, and 10.0 by controlling the pH value by 2 M HCl and 6 M NaOH. The liberated lactic acid was analyzed by HPLC, and the data were used for a calculation of % conversion efficiency. All experiments were performed in triplicate.

2.4. Inhibitory effect of lactic acid on PLA degradation

The effect of lactic acid concentration on PLA degradation was determined by adding various concentrations of lactic acid to the PLA degradation mixture. The reaction mixture contained 1 g of PLA powder, 25 mL of 200 U/mL crude enzyme, and various final concentrations of lactic acid (2000, 4000, 6000, and 8000 mg/L), and this reaction mixture was added into 100 mL Erlenmeyer flasks. The suspension was incubated at 60°C under shaking at 50 rpm for 48 h using an incubator shaker. The samples were taken every 6 h for evaluation, and the final lactic acid concentration was determined by HPLC. The control experiment was conducted using the reaction mixture described above without adding lactic acid. All experiments were carried out in triplicate.

2.5. Effect of the lactic acid removal method on PLA degradation

Lactic acid removal methods were investigated using a dialysis bag (Spectra Por membrane tubing, Thomas Scientific, U.S.A.). The experiments were separated into 3 processes as shown below:

2.5.1. Control experiment

PLA degradation was conducted without a dialysis step. The reaction mixture contained 1 g of PLA powder and 25 mL of 200 U/mL crude enzyme, and this reaction mixture was added into 100 mL Erlenmeyer flasks. The suspension was incubated at 60°C under shaking at 50 rpm for 48 h using an incubator shaker. The experiments were carried out in triplicate. The samples were taken every 6 h for evaluation and centrifuged at 10,000 × *g*, and the supernatant was used for determination of lactic acid concentration by HPLC. The weight loss of PLA powder was determined.

2.5.2. Separating PLA degradation and dialysis

One gram of PLA powder was added to 25 mL of 200 U/mL crude enzyme in 100 mL Erlenmeyer flasks. The reaction mixtures

were incubated at 60°C under shaking at 50 rpm for 24 h using an incubator shaker. All of the suspension was moved into a dialysis bag (Mw = 10 kDa cutoff), and the bag was soaked in a 250 mL beaker containing 200 mL of 100 mM Tris–HCl buffer pH 9.0; the beaker was incubated at 4°C for 24 h. The dialyzed enzyme was subjected to repeated degradation of PLA powder under the same conditions for 24 h. The experiments were carried out in triplicate. The samples were taken after degradation for evaluation, and the lactic acid concentration and PLA weight loss were determined.

2.5.3. Simultaneous PLA degradation and dialysis

One gram of PLA powder and 25 mL of 200 U/mL crude enzyme were taken in a dialysis bag. The dialysis bag was soaked in a 250 mL beaker containing 200 mL of 100 mM Tris–HCl buffer pH 9.0; the beaker was incubated at 60°C under shaking at 50 rpm for 48 h using an incubator shaker. The buffer was changed every 24 h. The degradation products were obtained after centrifugation at 10,000 × *g* at 4°C for 5 min. The experiments were performed in triplicate. The lactic acid concentration was determined by using HPLC. The percentage of PLA weight loss was analyzed.

2.6. Commercial PLA material degradation by crude PLA-degrading enzyme

One gram of PLA material, PLA tray (90% polylactic acid, size of 0.3 × 1 cm and thickness of 0.1 mm), and 25 mL of 200 U/mL crude enzyme were taken in a dialysis bag, which was soaked in a 250 mL beaker containing 200 mL of 100 mM Tris–HCl buffer pH 9.0; the beaker was incubated for 48 h at 60°C under shaking at 50 rpm using an incubator shaker. The buffer was changed every 24 h. The degradation products were obtained after centrifugation at 10,000 × *g* at 4°C for 5 min. All experiments were performed in triplicate. The lactic acid concentration was determined by HPLC. The percentage of PLA weight loss was analyzed.

2.7. Analytical method

The activity of the PLA-degrading enzyme was measured by the method previously reported by Youngpreda et al. [21]. The lactic acid concentration in the reaction mixture was analyzed by HPLC (Shimadzu, Japan) (InertSustain C18 column with a mobile phase of 10 mM $NH_4H_2PO_4$ (pH 2.6), flow rate of 1.0 mL/min, and temperature of 40°C). The percentage of PLA converted to release lactic acid (% conversion efficiency) was calculated according to the method of Youngpreda et al. [21]. The weight loss was determined by drying the residual PLA at 100°C overnight.

3. Results and discussion

3.1. Factors affecting PLA degradation by a crude enzyme produced by A. keratinilytica strain T16-1 in a 5 L stirred tank bioreactor

In this work, we focused on the scale-up of PLA degradation in a 5 L bioreactor. The results showed that the PLA degradation at agitation speeds of 50 and 100 rpm demonstrated lactic acid concentrations of 4627 and 2679 mg/L, respectively, at 60°C after incubation for 60 h. The percentage conversion efficiency at agitation speeds of 50 and 100 rpm was 25 and 14, respectively, as shown in Fig. 1. The lactic acid concentration at an agitation of 50 rpm was higher than that at 100 rpm by approximately 1.7-fold. These results concluded that increasing the mixing speed could decrease the PLA degradation efficiency due to the shear force of the disc turbine effect on the enzyme activity. It has been reported that enzymes are susceptible to mechanical force, which may disturb the elaborate shape of the complex molecules severely enough to cause denaturation of the molecules [22]. High agitation speeds were not preferred for the enzymatic activity, probably due to the shear stress caused by the impeller, which increases with the revolution speed [23].

The effect of pH on the PLA degradation efficiency was determined. The results showed that PLA degradation at different pH (8.0, 9.0, and 10.0) released lactic acid at concentrations of 16,651, 9092, and 7252 mg/L, respectively, and the % conversion was 89, 39, and 48, respectively, at a 50 rpm agitation rate and 60°C, as shown in Fig. 2. The highest PLA conversion efficiency was obtained at pH 8.0 after a 72 h reaction time. The lactic acid concentration at pH 8.0 was higher by approximately 2.3 and 1.8 times at pH 9.0 and 10.0, respectively. The optimum pH of purified PLA-degrading enzyme produced by the T16-1 strain was previously reported as 10.0 [8]. However, the stability of the enzyme might be decreased after an extended degradation time of PLA under extreme conditions such as high pH or temperature. In addition, the optimum pH of the three novel PLA-degrading enzymes from *A. orientalis*, named PLAase I, II, and III, were 9.5, 10.5, and 9.5, respectively [24]. The optimum pH of the PLA-degrading enzyme produced by *Laceyella sacchari* strain LP175 was 9.0 at 60°C [11].

3.2. Effect of the lactic acid concentration on PLA degradation

The effect of lactic acid on the PLA degradation was studied at different concentrations (0–8000 mg/L). The initial pH after addition of lactic acid at different lactic acid concentrations (0–8000 mg/L) was 9.0, 7.43, 6.85, 4.12, and 3.80. The result showed the % conversion

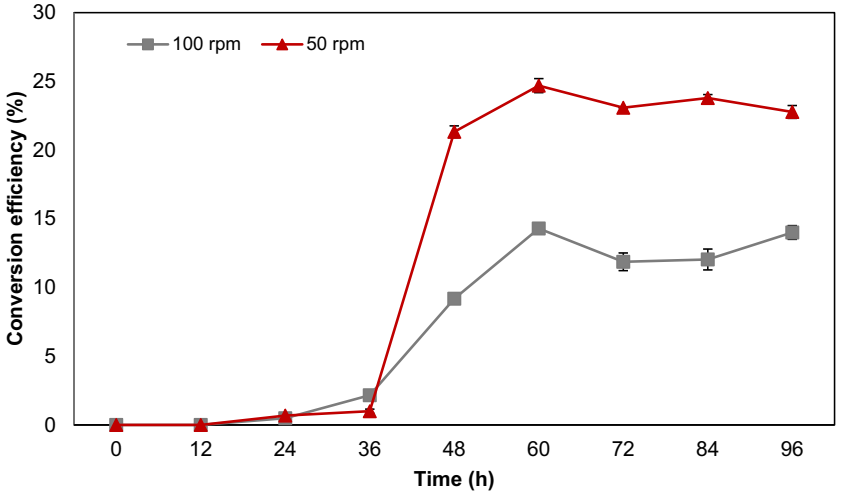

Fig. 1. Effect of agitation speed on PLA degradation by enzyme produced by *A. keratinilytica* strain T16-1 in a 5 L stirrer bioreactor under batch conditions of pH 9.0 and 60°C.

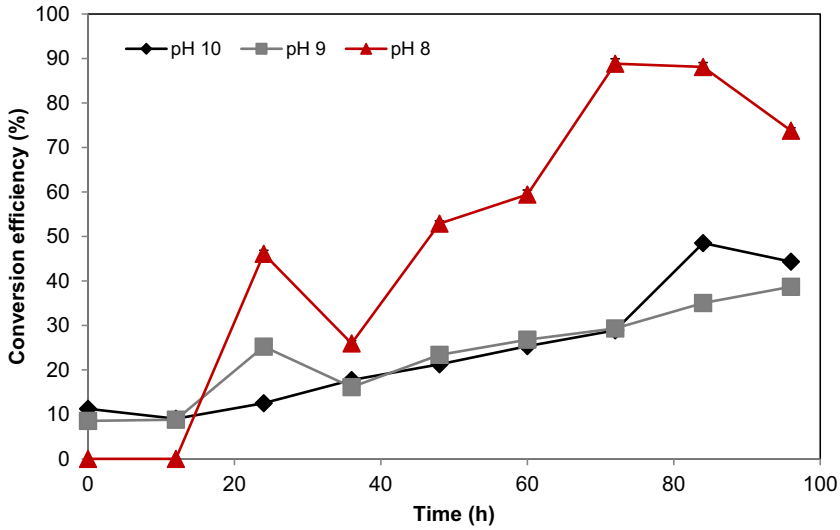

Fig. 2. Effect of controlled pH on PLA degradation by enzyme produced by *A. keratinilytica* strain T16-1 in a 5 L stirrer bioreactor under batch conditions of 50 rpm of agitation speed and controlled temperature at 60°C.

efficiency was 60, 28, 14, 6, and 5 at an initial lactic acid concentration of 0, 2000, 4000, 6000, and 8000 mg/L, respectively, as shown in Fig. 3. The degradation product was decreased when increasing the initial lactic acid concentration. This phenomenon indicated that lactic acid acted as an inhibitor of the enzyme and affected the PLA degradation process because lactic acid is the product of the enzyme reaction and by binding to the enzyme, it inhibits enzyme activity [25]. Moreover, the initial pH of the PLA degradation process at different lactic acid concentrations was very low (acidic), and this could have affected the enzyme activity, while the optimum pH for PLA degradation by the enzyme produced by *A. keratinilytica* was reported to be high (alkaline) [21]. Therefore, to enhance the degradation efficiency, the product inhibition effect should be considered.

3.3. Evaluation of the degradation method for the elimination of lactic acid during PLA degradation

The method for elimination of lactic acid during PLA degradation was studied by the dialysis technique. Using different methods, the results indicated that the percentage conversion efficiency was 62, 66, and 99 and the percentage of PLA powder weight loss was 14.75%, 25.95%, and 99.93% with experiments A, B, and C, respectively, as shown in Fig. 4. The highest % conversion efficiency and weight loss were obtained after incubation in the reaction mixture for 24 and 48 h, respectively, and by using method C. Therefore, the most effective method for lactic acid elimination was experiment C with simultaneous PLA degradation and dialysis. The lactic concentration of experiment C was 18,018 mg/L, which was higher than those in experiments A (11,685 mg/L) and B (12,318 mg/L) by approximately

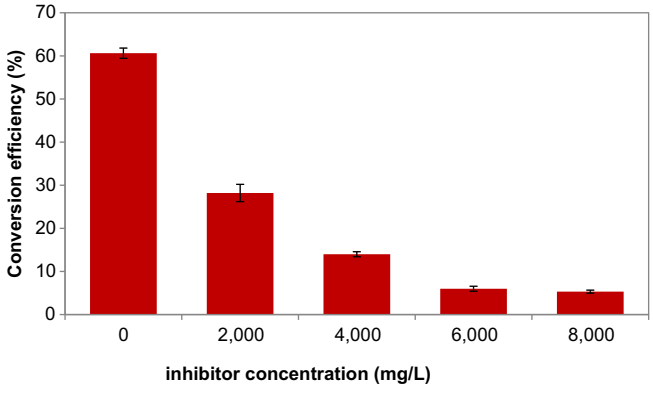

Fig. 3. Effect of lactic acid concentration as a degradation inhibitor on PLA degradation at 60°C after incubation for 24 h.

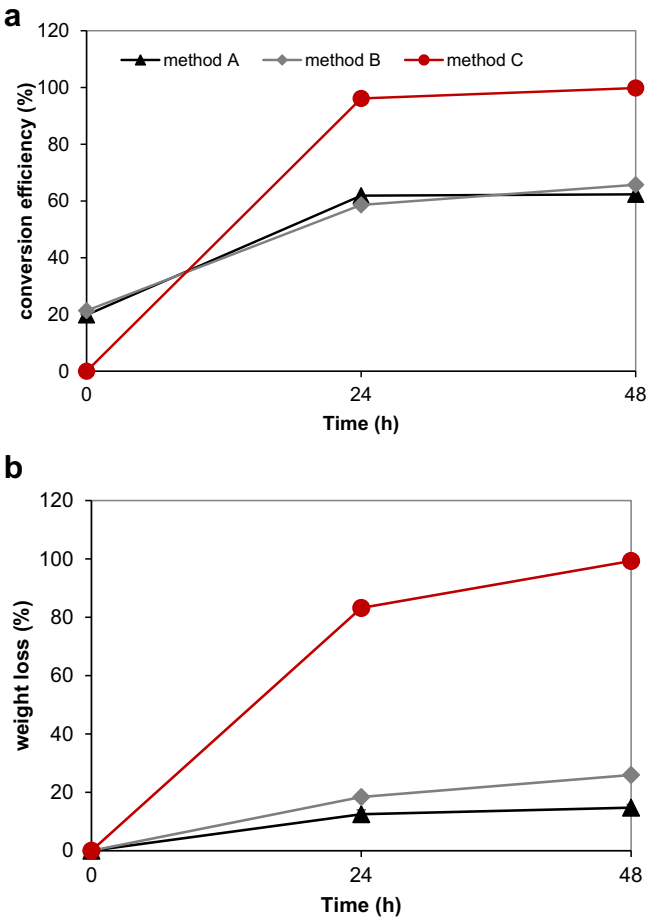

Fig. 4. Time course of PLA degradation using crude PLA-degrading enzyme produced by *A. keratinilytica* strain T16-1 by different lactic acid elimination methods. (a) Percentage of conversion efficiency; (b) Percentage of weight loss. Method A: control (without dialysis); Method B: separate PLA degradation and dialysis method; Method C: simultaneous PLA degradation and dialysis method.

Table 1
Comparison of percent PLA weight loss of PLA powder and commercial PLA material degradation using crude PLA-degrading enzyme with/without simultaneous degradation and dialysis.

	PLA powder		PLA tray	
	(Without dialysis)	Dialysis	(Without dialysis)	Dialysis
Weight loss (g)	0.1475 ± 0.02	0.993 ± 0.06	0.05 ± 0.02	0.32 ± 0.04
Weight loss (%)	14.75 ± 0.52	99.30 ± 0.68	5.00 ± 0.5	32.00 ± 0.76

All experiments were performed in triplicate, and the results were expressed as mean ± SD.

1.54- and 1.46-fold, respectively. It could be summarized that the simultaneous PLA degradation and dialysis method had the potential to improve PLA degradation by reducing lactic acid (byproduct) at the same time as the degradation reaction. Because the membrane pore size of the dialysis bag was 10 kDa, which was smaller than the Mw of the PLA-degrading enzyme (30 kDa), the enzyme was trapped inside the dialysis bag [8]. Further, lactic acid diffused out of the dialysis bag because it was smaller than the membrane pore size. Various previous reports have demonstrated the advantages of the dialysis bag. The dialysis method was used for recycling of homogeneous AU-DENs (Au_{100}-dendrimer-encapsulated nanoparticles) catalysts in the reduction of 4-nitrophenol [26]. Moreover, hydrosols of carbon nanotubes/graphene oxide (CNTs/GO) sealed in the dialysis bags were utilized for the highly efficient and re-pollution-free removal of trace Gd(III) from wastewater [27]. However, this is the first report of enhancing PLA degradation using the simultaneous PLA degradation and dialysis method.

3.4. Commercial PLA material degradation by a crude PLA-degrading enzyme

We evaluated commercial PLA tray degradation by a crude PLA-degrading enzyme produced by *A. keratinilytica* strain T16-1. Table 1 summarizes the percentage weight loss of PLA powder and PLA tray degradation by the crude enzyme. The results revealed that the PLA tray showed higher percent weight loss when it was degraded by the simultaneous degradation and dialysis method, with a weight loss of 32%. Furthermore, the control experiment showed only 5% weight loss. Thus, this present work presents a potential method for PLA degradation that can be applied for the degradation of PLA powder and a commercial PLA material in the future.

4. Conclusion

PLA degradation by an enzyme produced by *A. keratinilytica* strain T16-1 in a 5 L stirred tank bioreactor under batch conditions was investigated in this study. Results indicated that the best condition for PLA degradation was an agitation speed of 50 rpm at 60°C under a controlled pH of 8.0. The maximum percentage conversion efficiency was 89% after incubation for 72 h. Moreover, the effect of lactic acid as a product inhibitor was examined. The percentage PLA conversion efficiency was decreased the initial lactic acid concentration was increased. Thus, all the methods to remove lactic acid during PLA degradation were evaluated to determine the best method. Finally, the simultaneous PLA degradation and dialysis method showed the highest percent weight loss in both reactions, that is, PLA powder and commercial PLA tray.

Conflict of interest

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

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