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Research article

Solid state fermentation for poly (L-lactide)-degrading enzyme production by *Laceyella sacchari* LP175 in aerated tray reactor and its hydrolysis of poly (lactide) polymer

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Abstract

Poly(L-lactide) (PLLA)-degrading enzyme was produced by *Laceyella sacchari* LP175 under solid state fermentation in a static tray reactor using agricultural products of cassava chips and soybean meal as the substrate. The maximum enzyme production was obtained at 518 ± 8.5 U/g dry solid after cultivation at 50°C in moistened air with an aeration rate of 0.8 L/min for 24 hr followed by no aeration for 48 hr. Enzyme production increased with discontinuous aeration under solid state fermentation by maintaining the cultivation moisture content at a high temperature. Crude enzyme extracted from the fermented solid substrate was optimized at pH 9.0 and using 0.2 M of Tris-HCl buffer concentration for degradation of 100 g/L PLA polymer film, yielding 63.00 ± 4.59% after incubation at 50°C for 48 hr. Scaled-up hydrolysis in a 2.0 L stirrer batch reactor produced maximum degradation of 68.00 ± 2.5% at an agitation rate of 200 rpm. Enzyme production by *L. sacchari* LP175 in the aerated tray reactor was improved using agricultural products as substrates and hydrolysis of PLA polymer film at high concentration was enhanced, showing the method's potential for industrial application in the future.

Introduction

Polylactides (PLAs) obtained from the polymerization of L- and D-lactic acids at different ratios by the condensation process have interesting applications in various fields (Butbunchu and Pathom-Aree, 2019). PLA polymers can be used to replace other non-degradable petroleum-based plastic materials (Nampoothiri et al., 2010). PLAs are biodegradable but composting requires 50–90 d under controlled conditions for the temperature, moisture content and pH of the composting environment (Nampoothiri et al., 2010; Balakrishnan et al., 2011).

Numerous strains of microorganisms can produce poly(L-lactide)-degrading enzymes (PLLA-degrading enzymes) including *Amycolatopsis* sp., *Actinomadura* sp., *Streptomyces* sp., *Pseudomonas* sp. and *Laceyella* sp. (Nakamura et al., 2001; Jarerat et al., 2006; Panyachanakul et al., 2017; Bubpachat et al., 2018; Sriyapai et al., 2018). However, few studies have applied solid state fermentation (SSF) for enzyme production using various microorganisms.

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The advantages of SSF include the low cost of substrates, easier to up-scale than submerged culture, high productivity and less wastewater produced during the fermentation process (Heck et al., 2005; Krishna, 2005; Umsza-Guez et al., 2011; Zhang et al. 2015; Arora et al., 2018). Low-cost agricultural products of cassava chips and soybean meal used for PLLA-degrading enzyme production in a static tray bioreactor without aeration and using *L. sacchari* LP175, yielded 472 U/g dry solid after incubation at 50°C for 3 d (Lomthong et al., 2020). Therefore, the current study assessed the improvement of enzyme production with aeration supply to the reactor. Aeration is an important factor in SSF. Appropriate aeration supply to the SSF reactor has been reported to improve heat transfer and air flow through the substrate, with increased growth and enzyme production (Fujian et al., 2002; Arora et al., 2018; Khanahmadi et al., 2018).

PLA polymers are degraded by various microbial enzymes under diverse conditions such as lipase from *Alcaligenes* sp. which hydrolyzed at 55°C and pH 8.5 (Hoshino and Isono, 2002) and alcalase from *Bacillus licheniformis* which hydrolyzed PLA polymer at 60°C and pH 9.5 (Lee et al., 2014). Lomthong et al. (2020) reported that PLA polymer film was approximately 20% hydrolyzed when incubated at pH 9.0 with the temperature controlled at 50°C for 24 hr, compared to the same conditions in the presence of crude PLLA-degrading enzyme produced by *L. sacchari* LP175. The main obstacle for hydrolysis of PLA polymer by crude enzyme was feedback inhibition from the obtained lactic acid, which reduced the pH and decreased enzyme hydrolysis efficiency (Lomthong et al., 2017). To solve this problem, the hydrolysis conditions such as pH and buffer concentration were studied to control the pH value of the reaction.

This research investigated the effect of aeration on PLLA-degrading enzyme production by *L. sacchari* LP175 under SSF. In addition, the study investigated the enhancement of PLA polymer film biodegradation at high concentration by the crude enzyme obtained from SSF.

Materials and Methods

Microorganism and inoculum preparation

The thermophilic filamentous bacterium, *L. sacchari* LP175 has been reported as a potent strain for the production of raw starch degrading enzyme and PLLA-degrading enzyme at high temperature (45–50°C), with the ability to utilize agricultural products as substrate for growth and enzyme production (Lomthong et al., 2015). The LP175 strain was deposited at the Thailand Institute of Scientific and Technological Research (TISTR) Bangkok MIRCEN culture collection as *L. sacchari* TISTR 2280 as described by Lomthong et al. (2015). A loop full of LP175 culture was grown in nutrient broth at 50°C for 24 hr. Cell pellets were obtained using centrifugation (10,000 revolutions per minute; rpm) at 4°C. Then, they were suspended in sterile 0.85% NaCl solution, adjusted to an optical density (OD) of 1.0 at a wavelength of 600 nm (1×10^7 colony forming units (CFU)/mL) and used as an inoculum for SSF.

Solid state fermentation

Solid state fermentation was performed in a static tray reactor using 100 g dry solids comprising 86 g of cassava chips and 14 g of soybean meal (Lomthong et al., 2020). The solid substrates were mixed with 0.1 g peptone and moistened with 185 mL of mineral salt solution at 2.0 cm of substrate thickness, as described by Lomthong et al. (2020). After sterilizing at 121°C for 15 min, 50 mL of *L. sacchari* LP175 inoculum was added to the solid substrate medium to make the final moisture content of 70%. The sterilized air flow rate was adjusted to 0.4–2.0 L/min with incubation at 50°C for 3 d. To minimize the evaporation rate, the air was saturated by passing the air inlet through a bottle containing sterile distilled water before entering the tray reactor. Fermentation without air flow was used as the control.

A discontinuous aeration supply strategy was operated by feeding the air flow rate at 0.4–2.0 L/min to the static tray reactor for 24 hr, containing the optimized combination of solid substrates, mineral salt solution and *L. sacchari* LP175 inoculum, as described above. Then, further cultivation was continued for 48 hr without aeration feeding. At the end of the fermentation period, 5.0 g of solid substrate was removed to determine the PLLA-degrading activity and the solid dry weight. Ten milliliters of Tris-HCl buffer (100 mM, pH 9.0) were added and allowed to stand at 4°C for 3 hr. A clear solution of crude enzyme was obtained using centrifugation at 5,000 rpm for 10 min at 4°C, followed by filtration through a 0.45 µm pore size membrane (EMD Millipore Millex™), as reported by Lomthong et al. (2020). The clear filtrate was used to determine the PLLA-degrading activity using 0.1% PLLA emulsion by adding 0.25 mL of the appropriate enzyme dilution to 2.25 mL of PLLA emulsion, followed by incubation at 60°C for 30 min, as described by Sukkhum et al. (2009). One unit of PLLA-degrading activity was defined as the decrease in the optical density by 1.0 unit at 630 nm per minute under the assay conditions described. Growth of LP175 was determined using a dilution plate count on nutrient agar plates, as described by Lomthong et al. (2020). The moisture content was determined by weight loss of solid substrate after storing at 100°C overnight.

Biodegradation of polylactide polymer film

Polymer film preparation

The PLA polymer film was prepared by dissolving 2.0 g of PLA pellets (NatureWorks; Minnetonka MN, USA) in 200 mL of dichloromethane (Merck, Germany). After the pellets had completely dissolved, the liquid solution was poured into a stainless steel tray, covered with aluminum foil and then dried overnight at room temperature. The PLA film was cut into sample sizes of 2.0 cm×2.0 cm and used to investigate the degradation ability of the PLLA-degrading enzyme produced by *L. sacchari* LP175 extracted from SSF.

Effect of polylactide polymer film concentration

PLA degradation was investigated in 250 mL Erlenmeyer flasks containing 50 mL of crude enzyme extracted from the solid substrate (100 U/mL). The PLA polymer films at various concentrations (5, 10, 20, 40, 80 and 100 g/L) were separated and added to the reaction. The reaction mixture was incubated in a shaking incubator at 150 rpm and

50°C for 48 hr. At the end of the reaction, the clear supernatant was used to determine the pH and dry weight of the retained PLA film. The PLA film residue was dried at 50°C for 12 hr and the weight loss and percentage degradation were determined according to Equation 1 below following Lomthong et al. (2015):

Degradation = (Initial film weight. ® Retained film weight) / Initial film weight) × 100 (1)

Effect of pH and buffer concentrations on polylactide polymer film degradation

The PLA polymer film degradation effects were investigated of Tris-HCl buffer (pH 8.0 and 9.0) and glycine-NaOH buffer (pH 10) at concentrations of 0.1, 0.2, 0.3 and 0.5 M. The crude enzyme obtained from SSF fermentation was washed three times with the different buffers as described above using Amicon® Ultra-15 Centrifugal Filter Units with a 10 kDa cutoff (Merck Millipore; Bedford, MA, USA). Each reaction was conducted in a 250 mL Erlenmeyer flask containing 50 mL of enzyme, which exchanged the pH and buffer concentration with PLA polymer film at 100 g/L. All experiments were incubated in a shaking water bath at 150 rpm and 50°C for 48 hr. The clear supernatant was used to determine the pH of the reaction, while the residue of PLA film dried at 50°C for 12 hr was used to measure the weight loss and to calculate the percentage degradation as described above.

Biodegradation of polylactide polymer film in 2.0 L stirrer fermenter

To study the feasibility for application in large-scale hydrolysis and to investigate the effect of the agitation rate on the hydrolysis of PLA polymer film, the reaction was operated in a 2.0 L stirrer fermenter with 1.0 L working reaction volume. Enzyme at optimum pH and buffer concentration was added to the fermenter with 100 g/L of PLA polymer film. The reaction was operated at 50°C for 48 hr with agitation rates of 50, 100, 200 and 300 rpm. The PLA film residue was dried at 50°C for 12 hr and the weight loss and percentage degradation were calculated as described above. A scanning electron microscope (model SU8020; Hitachi; Tokyo, Japan) was used to study the native bioplastic PLA polymer without treatment in the reactor and the residues after treatment with crude enzyme extracted from the fermented solid substrate of *L. sacchari* LP175 at 10 kV.

Statistical analysis

The results were reported as the mean (± SD) of three determinations (n = 3) and the data were analyzed using one-way analysis of variance (ANOVA) (SPSS software version 21.0, SPSS Inc., USA). Differences among mean values were tested using the Duncan's multiple range tests. Values were considered significant at $p < 0.05$.

Results and Discussion

Effect of aeration rate on poly(L-lactide)-degrading enzyme production under solid state fermentation

Aeration is an important factor for growth and enzyme production by *L. sacchari* LP175 as a means of heat transfer generated by the fermentation at high substrate thickness (Hanphakphoom et al., 2014; Lomthong et al., 2020). Each bacterial strain has its own physiological and metabolic system (Singh et al., 2012), and requirements of oxygen for growth and enzyme production vary (Lomthong et al., 2017). The effect of aeration rates on growth and PLLA-degrading enzyme production by *L. sacchari* LP175 under SSF are shown in Fig. 1.

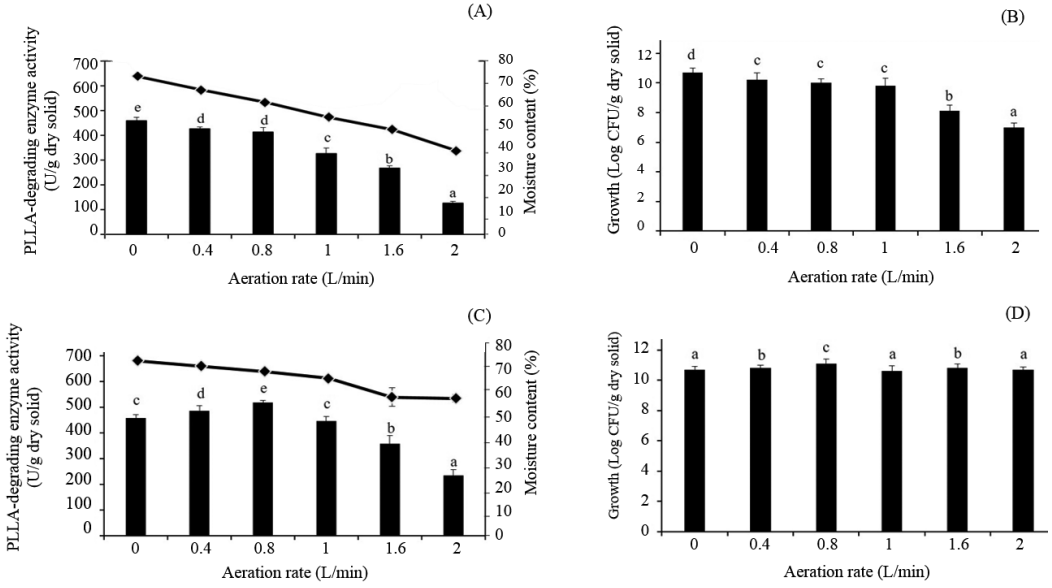

Fig. 1 Effect of aeration rate on growth and poly(L-lactide) (PLLA)-degrading enzyme production by *Laceyella sacchari* LP175 under solid state fermentation: (A, B) continuous aeration supply strategy; (C, D) discontinuous aeration supply strategy, where error bars = ± SD; different lowercase letters above columns indicate significant ($p < 0.05$) difference among means.

The supply of aeration to the fermentation reactor for 72 hr had a negative effect on enzyme production and growth (Fig. 1A, 1B) compared to fermentation without aeration feeding. The supply of continuous aeration to the reactor disrupted enzyme production and decreased the utilization ability of the substrate. The supply of excess aeration for a long time period induced the removal of water content and metabolic heat from solid substrates under SSF (Khanahmadi et al., 2018). Arora et al. (2018) also reported that aeration in a solid state reactor affected the growth of microorganisms and enzyme production but that oversupply of the aeration rate to the reactor decreased enzyme production under SSF. In the current study, *L. sacchari* LP175 was cultivated at high temperature (50°C), and this stimulated the evaporation rate of moisture. Maintaining the moisture content of the solid substrate in the reactor requires high relative humidity in the incubator which increases the cost of enzyme production at this stage. Therefore, to avoid a decrease in the moisture content of the solid substrate that negatively affected PLLA-degrading enzyme production by *L. sacchari* LP175 under SSF, a discontinuous aeration supply strategy was adopted to increase enzyme production.

The aeration feeding strategy was performed at different aeration rates for 24 hr and then for 48 hr without aeration, as described above. The results showed that aeration at 0.8 L/min for 24 hr and further cultivation for 48 hr without aeration produced the highest enzyme production at 518 ± 8.5 U/g dry solid (Fig. 1C). At higher aeration of 1.0 L/min, the reduced substrate fermentation was attributed to low water activity (a_W), while shear stress damaged the filamentous microorganisms (Arora et al., 2018). The aeration rate affected growth and PLLA-degrading enzyme production by *L. sacchari* LP175 in an airlift fermenter under submerged fermentation (Lomthong et al., 2017), with a decrease of 50% when the aeration rate increased by up to 1.5 times. Singh et al. (2007) reported that rates of oxygen transfer played an important role in overall microbial metabolism but that different microorganisms behaved differently under various conditions of oxygen supply. A low aeration rate supplied to the solid culture reduced the air flow through the substrate; consequently, there was a decreased oxygen uptake rate (Fujian et al., 2002; Arora et al., 2018). In the current study, the use of a discontinuous aeration supply strategy (first 24 hr aeration and then 48 hr without aeration) stimulated growth and PLLA-degrading enzyme production of the aerobic bacterium, *L. sacchari* LP175 (11.1 ± 0.3 log CFU and 518 ± 8.5 U/g dry solid, respectively). This strategy was simple and showed the advantage for upscaling at an industrial level, since the non-requirement for continuous aeration reduced energy consumption and operation costs.

A summary of PLLA-degrading enzyme production by *L. sacchari* LP175 under SSF is shown in Table 1. Application of discontinuous aeration supply improved enzyme production compared to the previous study and can be used as a model for future development at the industrial level.

Biodegradation of polylactide polymer film

Effect of polylactide polymer film concentration

Among numerous kinds of degradable polymers, PLA is currently the most promising and popular and is considered as a green, eco-friendly material (Nampoothiri et al., 2010). The results of PLA polymer film biodegradation by PLLA-degrading enzyme produced from *L. sacchari* LP175 are shown in Fig. 2. At 5 g/L and 10 g/L of PLA film, the PLLA-degrading enzyme produced from *L. sacchari* LP175 generated complete hydrolysis (100% degradation) when incubated at 50°C for 48 hr, while at 100 g/L, the percentage degradation decreased to 48.9 ± 2.3%. The decrease in degradation was due to inhibition of enzyme activity by liberated lactic acid in the reaction, as previously reported by Lomthong et al. (2017), who noted that 0.8 g/L of lactic acid inhibited 50% of the enzyme activity. Furthermore, at high concentrations, lactic acid decreased the pH in the reaction and enzyme activity decreased. Therefore, to solve this problem, the optimum conditions for the hydrolysis of PLA polymer at high concentration were studied in further experiments.

Effect of pH and buffer concentrations on polylactide polymer film degradation

The maximum degradation (63.00 ± 4.59%) resulted from hydrolysis in 0.2 M Tris-HCl buffer at pH 9.0, as shown in Table 2. The buffer concentration (pH 9.0) at 0.1 M produced 54.70 ± 1.76% degradation, which was lower than at 0.2 M because the liberated lactic acid from the hydrolysis lowered the pH of the reaction and subsequently reduced enzyme activity. Each enzyme has an optimum pH for PLA degradation. For example, *Amycolatopsis orientalis* ssp. *orientalis* produced optimum degradation in 50 mM of glycine-NaOH buffer at pH 10.0. This completely degraded 4.0 g/L of PLA granules after 5 d of incubation (Li et al., 2013).

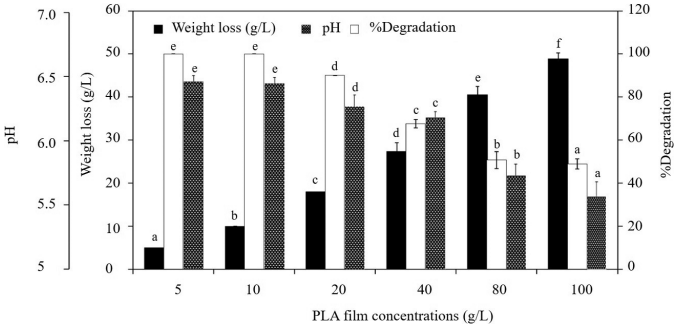

Fig. 2 Effect of polylactide polymer (PLA) film concentration for degradation by poly(L-lactide)-degrading enzyme produced by *Laceyella sacchari* LP175 at 50°C for 48 hr, where error bars = ± SD; different lowercase letters above columns representing weight loss or pH or % degradation indicate significant ($p < 0.05$) difference within each parameter.

Table 1 Poly(L-lactide)-degrading enzyme production by *Laceyella sacchari* LP175 under solid state fermentation (SSF) with different aeration conditions

Condition	Enzyme activity (U/g dry solid)	Reference
SSF in static tray reactor without aeration for 72 hr at 50°C	472 ± 9.8[b]	Lomthong et al., 2020
SSF in static tray reactor with 0.8 L/min aeration for 72 hr at 50°C	413 ± 7.8[a]	Current study
SSF in static tray reactor with 0.8 L/min aeration for 24 h and without aeration for 48 hr at 50°C	518 ± 8.5[c]	Current study

Table 2 Effect of pH and Tris-HCl buffer concentration on mean degradation percentage ± SD of polylactide polymer film (100 g/L) by poly(L-lactide)-degrading enzyme from *Laceyella sacchari* LP175

Run	pH	Buffer concentration (M)	Degradation (%)
1	8	0.1	25.17 ± 1.48[a]
2	8	0.2	39.63 ± 0.85[c]
3	8	0.3	42.00 ± 4.24[d]
4	8	0.5	34.40 ± 0.92[b]
5	9	0.1	54.70 ± 1.76[g]
6	9	0.2	63.00 ± 4.59[i]
7	9	0.3	58.60 ± 4.24[h]
8	9	0.5	48.60 ± 1.41[f]
9	10	0.1	48.00 ± 5.65[f]
10	10	0.2	45.25 ± 1.69[e]
11	10	0.3	39.42 ± 1.48[c]
12	10	0.5	38.90 ± 2.19[c]

The buffer concentration retarded the change in pH during hydrolysis. However, as previously reported, 20% weight loss of PLA polymer was degraded without enzyme at pH 9.0 (Lomthong et al., 2020). Normally, PLA degradation relates to chemical hydrolysis due to cleavage of the polymer chain during hydrolytic degradation, as explained by Elsawy et al. (2017). The hydrolysis of PLA occurs by random cleavage of the ester bonds under basic conditions, depending on the surrounding moisture environment and diffusion coefficients of the chain fragments within the polymer (Schliecker et al., 2003).

Biodegradation of polylactide polymer film in a 2.0 L stirrer fermenter

The effects of the agitation rate on the percentage degradation of PLA polymer film were investigated to scale up the hydrolysis, with the results shown in Fig. 3. The maximum degradation at 68.00 ± 2.5% was obtained at 200 rpm, which was higher than for hydrolysis using a shaking flask. Panyachanakul et al. (2019) reported that agitation was an important factor for the hydrolysis of PLA polymer in a 5.0 L stirrer fermenter using the PLA-degradation enzyme produced by *A. keratinilytica* strain T16-1. At 50 rpm the highest percentage degradation was recorded, yielding 89% using 4 g/L of polymer substrate when incubated at 60°C for 72 hr. In the current study, low agitation speeds (50–100 rpm) resulted in a lower degradation efficiency due to the limitation of enzyme contact with the surface of the PLA polymer film as a result of mixing problems at high substrate concentration. At high mixing speeds, the degradation efficiency decreased due to the shear forces of the disc turbine effect on enzyme activity that caused denaturation of the enzyme molecules (Panyachanakul et al., 2019). Scanning electron micrographs revealed that the PLA polymer film was affected by loss of rigidity, with the appearance of fractures in PLA films compared to PLA film without hydrolysis by PLLA-degrading enzyme produced from *L. sacchari* LP175 (Fig. 4). This showed the feasibility of upscaling enzyme hydrolysis with high concentrations at an industrial level in the future.

High production of PLLA-degrading enzyme by *L. sacchari* LP175 (518 ± 8.5 U/g dry solid) was achieved under SSF using a discontinuous aeration supply strategy. The optimum levels for the pH and buffer concentration enhanced the degradation of the PLA polymer film at high concentration (100 g/L). The current findings can be applied to reduce the environmental accumulation of PLA polymer as a biomodel for sustainable green technology.

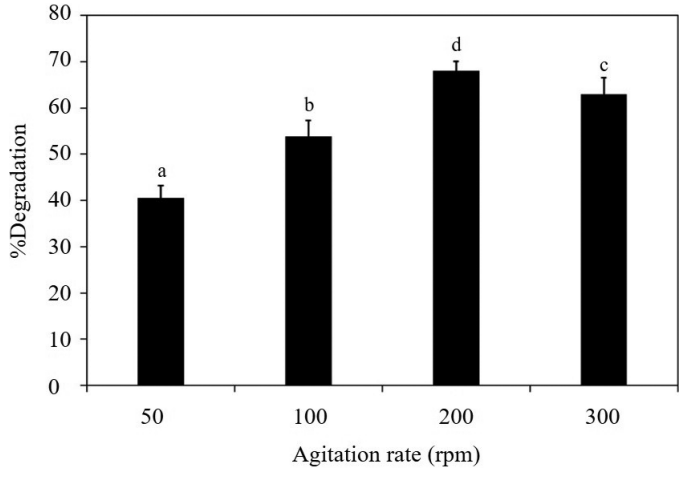

Fig. 3 Effect of agitation rate on degradation percentage of polylactide polymer film in a 2.0 L stirrer fermenter at 50°C for 48 hr, where error bars = ± SD; different lowercase letters above columns indicate significant ($p < 0.05$) difference among means.

Conflict of Interest

The authors declare that there are no conflicts of interest.

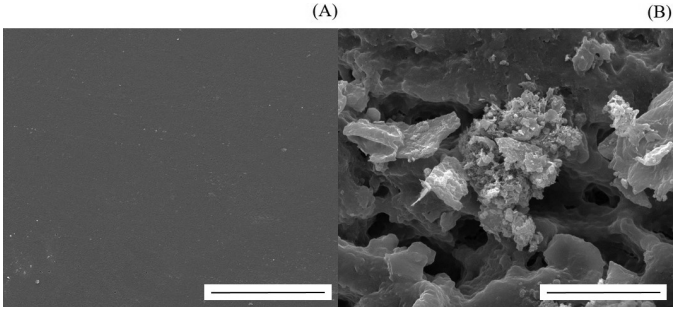

Fig. 4 Scanning electron micrographs of native (A) and digested polylactide polymer film (B) by poly(L-lactide)-degrading enzyme produced by *Laceyella sacchari* LP175 after hydrolysis at 50°C for 48 hr, where scale bar = 10μm

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