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Research article

# Poly (L-lactic acid)-degrading activity from endophytic *Micromonospora* spp. and catalytic analysis using surface plasmon resonance

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**Abstract**

Poly (L-lactic acid) (PLA) is one of the biodegradable plastics that has been favorably consumed in substitution of petroleum-based plastics. PLA can be degraded by microorganisms including actinomycetes. In an attempt to seek for PLA degradable actinomycetes, three of the 164 strains of endophytic actinomycetes from the Genetics-Microbiology Kasetsart University (GMKU) culture collection showed clear zone formation on PLA-emulsified agar. 16S rRNA gene sequencing of the positive strains revealed that they belonged to the genus *Micromonospora*. After incubation of these strains on PLA film in basal medium for 2 wk, the films had clearly deteriorated when viewed under a scanning electron microscope. The degradation of the PLA by crude enzymes of these strains was investigated using surface plasmon resonance (SPR) analysis. A crude enzyme (*Micromonospora* sp. GMKU 358) produced the highest activity with a specific activity of 0.22 mg/hr/mg crude enzyme at pH 10 without ionic strength (no sodium chloride). This was the first report of the PLA-degrading activity of an endophytic *Micromonospora* characterized using SPR.

## Introduction

The production of bioplastic has been predicted to increase from 4.2 million t in 2016 to 6.1 million t in 2021 (Schwede, 2016). There is an upward trend on the production capacity of poly (L-lactic acid) (PLA) and other starch-based plastics from 0.9 million t to 1.3 million t between 2016 and 2021 (Schwede, 2016). PLA has more suitable properties (including high tensile strength and modulus) for utilization than other bio-based plastics (Tabasi and Ajji, 2015; Emadian et al., 2017).

PLA is an aliphatic polyester biodegradable plastic that has replaced some petroleum-based plastics because the latter are causing global environmental problems. PLA has been produced from renewable agricultural resources such as corn starch and sugar cane using lactic acid bacterial fermentation and can be degraded by microbes (Li and Cui, 2010). The majority of PLA-degrading microbes belong to the actinomycetes and include: *Amycolatopsis*, *Actinomadura*, *Kibdelosporagium*, *Laceyella*, *Lentzea*, *Micromonospora*, *Nonomureae*, *Saccharothrix*, *Streptoalloteichus* and *Thermoactinomyces* (Pranamuda et al., 1997; 2001; Ikura and Kudo, 1999; Nakamura et al., 2001; Jarerat et al., 2002; Jarerat and Tokiwa, 2003; Tokiwa and Jarerat, 2003; 2004; Sukkhum et al., 2009; 2011; Chomchoei et al., 2011; Hanphakphoom et al., 2013). Other groups of bacteria have been found, such as *Bacillus brevis*, *Bacillus licheniformis*, *Bortetellapetrii*, *Burkholderiacapacia*, *Cryptococcus* sp.,

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*Geobacillus thermocatenulatus*, *Paenibacillus amylolyticus* and *Pseudomonas tamsuii* (Tomita et al., 1999; Akutsu-Shigeno et al., 2003; Teeraphatpornchai et al., 2003; Tomita et al., 2004; Masaki et al., 2005; Kim et al., 2008; Wu, 2009; Kim and Park, 2010; Liang et al., 2016). Furthermore, PLA-degrading activity has been reported from fungi such as *Aspergillus niger*, *Cladosporium* sp., *Doratomyces microsporus*, *Fennellomyces linderi*, *Fusarium* sp., *Lecanicillium saksenae*, *Mortierella* sp., *Penicillium roquefort*, *Purpureocillium* sp., *Pseudozyma antarctica*, *Rhizopus delemer* and *Verticillium* sp. (Fukuzaki et al., 1989; Torres et al., 1996; Nakajima-Kambe et al., 2012; Shinozaki et al., 2012; Karamanlioglu et al., 2014; Penkhrue et al., 2015).

Previously, biodegradable plastic-degrading enzymes with PLA-degrading activity were determined based on clear zone formation (Tokiwa et al., 1999), film weight loss and monomer production (lactic acid) (Pranamuda et al., 1997; Ikura and Kudo, 1999; Pranamuda et al., 2001; Jarerat and Tokiwa, 2001; 2003), turbidity (Nakamura et al., 2001) and change in molecular weight and viscosity (Tomita et al., 1999; 2003; 2004). Recently, surface plasmon resonance (SPR) has been used to measure the direct mass change with high sensitivity for the enzymatic degradation of the polymer films (Sumner et al., 2000). For example, SPR was used to demonstrate the biodegrading activity of thermophilic PHA polymerase (PhaZ-Th) from *Pichia pastoris* catalyzed from poly [(R)-3-hydroxybutyrate] (PHB) film (Phithakrotchanakoon et al., 2009), recombinant polyester-degrading hydrolase from *Thermobifida* sp. BCC23166 (rTfH) overexpressed by *Streptomyces rimosus* targeted on aliphatic-aromatic co-polyester (Sinsereekul et al., 2010) and cutinase-like enzyme from *Pseudozyma antarctica* JCM10317 degrading polyester film (Shinozaki et al., 2013).

The current study screened endophytic actinomycetes for their PLA-degrading activity based on clear zone formation. PLA film degradation by positive strains was observed under a scanning electron microscope. The PLA-degrading activity from crude enzymes was subjected to SPR analysis.

## Material and Methods

### *Poly (L-lactic acid) film and poly (L-lactic acid) medium preparation*

PLA film was prepared by dissolving a 50 mg of PLA pellet (MW $43\times10^3$, vyloecol BE-400 PLA; TOYOBO; Japan) in 50 mL of dichloromethane and pouring onto a glass plate, followed by air drying overnight at room temperature. The fabricated PLA films were cut into 1 $cm^2$ pieces and surface sterilized by soaking in 70% ethanol for 1 min and then air dried before use.

Emulsified PLA medium was prepared by dissolving 1 g of PLA pellet in 40 mL of dichloromethane. The solution was added into sterilized 1 L basal medium [1 g yeast extract, 4 g $(NH_4)_2SO_4$, 2 g $K_2HPO_4$, 1 g $KH_2PO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, pH 7.0] and sonicated with an ultrasonic processor (model VCX130; Sonic and Materials, Inc.; USA) for 5 min. For preparation of emulsified PLA agar, 15 g/L of agar was added (Sukkhum et al., 2009).

### *Screening for poly (L-lactic acid)-degrading activity by clear zone formation*

Endophytic actinomycetes from the Genetic-Microbiology Kasetsart University (GMKU) culture collection (Indananda, 2013) were grown on tryptic soy broth agar (TSA) at 28°C for 7 days. A loop full of actinomycete culture was transferred into 10 mL of tryptic soy broth (TSB) and incubated at 28°C and 200 revolutions per minute (rpm) for 7 d. Cells were harvested using centrifugation at 5,000 rpm for 5 min and washed twice with sodium phosphate buffer at pH 7.0. Next, cells were resuspended in 10 mL of basal medium and 10 µL of cell suspension was dropped on emulsified PLA agar medium and incubated at 28°C up to 7 d. The degradation ability was determined by a clear zone formation around the colony (Sukkhum et al., 2009).

### *Identification of poly (L-lactic acid)-degrading actinomycetes by 16S rRNA gene sequencing*

Total DNA samples of PLA-degrading actinomycetes were prepared as described by Kieser et al. (2000). Briefly, 16S rRNA gene was amplified using specific primers: STR1F (5' TCACGGAGAGTTT GATCCTG 3') and STR1530R (5' AAGGAGATCCAGCCGCA 3') (Kataoka et al., 1997) using the polymerase chain reaction (PCR) conditions described by Rachniyom et al. (2015). The PCR products were purified and subsequently sequenced at Macrogen (Korea). The 16S rRNA gene sequence of each strain was compared to the type strains in the database using EzBioCloud (Yoon et al., 2017). Multiple alignments of 16S rRNA gene sequences of PLA-degrading strains and closely related type strains were performed using ClustalW (Thompson et al., 1994). A phylogenetic tree was constructed using the neighbor-joining method in the MEGA version 6.0 software package (Tamura et al., 2013) with bootstrapping based on 1,000 replicates.

### *Degradation of poly (L-lactic acid) film in liquid culture*

Degradation of PLA film was examined using the modified method of Sukkhum et al. (2011). PLA-degrading strains were grown in TSB at 28°C and 200 rpm for 7 d. Cells were washed with basal medium twice and resuspended in 1 mL basal medium. A 10% (volume per volume) solution of cell suspension was inoculated into 50 mL basal medium in Erlenmeyer flasks containing three PLA films and incubated at 28°C and 200 rpm for 2 wk. Basal medium was used as the negative control. Then, the PLA films were washed with distilled water and air-dried overnight. The films were coated with gold using a Polaron Range (Model SC7620; Kent, UK) and the surface of each PLA film sample was observed using a scanning electron microscope (Model LEO1450 VP; Carl Zeiss AG, Jena, Germany). The culture broths of positive strains were further enzymatically analyzed for PLA-degrading activity using surface plasmon resonance.

*Surface plasmon resonance analysis*

*Surface plasmon resonance configuration*

The surface plasmon resonance (SPR) used was constructed by the Electronics and Computer Technology Center, Bangkok, Thailand. PLA coated on a sensor chip (gold thickness of 50 nm on 10 nm of chromium) was set up on the prism with index matching fluid. The flow cell contained seven channels; channels 1–6 were used for the reaction chamber for enzyme degradation testing and the seventh channel was used as a negative control. A pump at 2 µL/min flow rate was used to inject crude enzyme over the sensor chip surface.

*Sensor chip preparation*

PLA solution was dissolved in chloroform and stirred overnight at room temperature. The sensor chips were sonicated in water followed by methanol immersion for 15 min, then dried with nitrogen gas. PLA was coated on the sensor chip using a spin coater (Model 6700D, Specialty Coating System; Rocklin, CA, USA) using centrifugation at 1,500 rpm for 60 s.

*SPR data analysis*

The running buffer was injected over the multi-channel sensor chip at 0.5 µL/min to set up the baseline. Crude enzymes were concentrated using ultrafiltration with an Amicon centrifugal unit (MWCO 10 kDa; Millipore; Burlington, MA, USA) in 0.1 M sodium phosphate buffer at pH 7. The protein concentration was analyzed using Bio-Rad Protein Assay Reagent based on Bradford's method (Bio-Rad; Hercules, CA, USA) using bovine serum albumin as a standard protein and measured by a spectrophotometer at an optical density (OD) of 595 nm. Each crude enzyme was injected into the sample channels. The flow was stopped and the crude enzyme was analyzed. The degradation rate was determined from the sensogram based on the initial slope, using the system calibration where an SPR angle shift of 1 millidegree correlated with a mass change of 1.05 ng/cm$^2$ (Phithakrotchanakoon et al., 2009). The unit of enzyme was calculated using Equation 1:

$$\text{Specific activity} = \text{Mass change} / (\text{Enzyme concentration} \times 5) \quad (1)$$

The specific activity was measured in nanograms per hour per milligram of enzyme unit, the volume of the chamber was 5 µL, the mass change was measured in nanograms per hour and the enzyme concentration was measured in micrograms per milliliter.

The experiments were carried out at room temperature (25°C). The effect of pH on PLA-degrading catalysis was studied in the pH range 3–6 in 0.2 M citrate buffer, in the pH range 6–8 in 0.2 M phosphate buffer and in the pH range 8–10 in 0.2 M Tris-HCl. The effect of ionic strength on PLA degradation was investigated with 0.05 M NaCl and 0.25 M NaCl, and without NaCl in suitable running buffer. The alteration of reflectivity was measured at various angles on the sensor chip for 60 min. The angle shift was in concordance with the reduction in the PLA mass on the sensor ship surface, which was converted into the mass change, and the reflected enzymatic degradation activity was recorded in the linear range of the SPR curve as a function of time. Each experiment was compared with negative controls without crude enzyme.

**Results and Discussion**

*Screening of poly (L-lactic acid)-degrading endophytic actinomycetes*

Three (GMKU 353, GMKU 358, GMKU 362) out of 164 endophytic actinomycetes from the GMKU culture collection were able to degrade emulsified PLA agar based on producing a clear zone formation after incubation at 28°C for 7 d (Fig. 1A). The 16S rRNA gene sequences of PLA-degrading strains were compared with the database and a phylogenetic tree was constructed (Fig. 2). The results indicated that strain GMKU 353 (GenBank accession no. JF699703) was closely related with *Micromonospora tulbaghiae* DSM 45142$^T$ (99.01%), whereas GMKU 358 (GenBank accession no. JF699704) and GMKU 362 (GenBank accession no. JF699705) were similar to *Micromonospora chalcea* DSM 43026$^T$ (99.44% and 99.30%, respectively). Qi et al. (2017) isolated several PLA-degrading strains from soil or water. However, the current study was the first to report that actinomycetes associated with plants carry PLA-degrading activity. These results agreed with other reports that most of PLA-degrading microbes belonged to actinomycetes (Pranamuda et al., 1997; 2001; Ikura and Kudo, 1999; Nakamura et al., 2001; Jarerat et al., 2002; Jarerat and Tokiwa, 2003; Tokiwa and Jarerat, 2003; 2004; Sukkhum et al., 2009; 2011; Chomchoei et al., 2011; Hanphakphoom et al., 2013). Based on the morphological study, three strains of PLA-degrading *Micromonospora* were reported to grow well on mannitol soya (MS; Hobbs et al., 1989), TSA and the International Streptomyces Project 3 (ISP 3; Shirling and Gottlieb, 1966) agar media. Colonies of the three strain are shown in Fig. 1B.

*Poly (L-lactic acid) film degradation by Micromonospora spp.*

After shaking incubation of PLA-films with *Micromonospora* spp. GMKU 353, GMKU 358 and GMKU 362 in basal medium at 28°C for 2 wk, the films showed clear deterioration compared to the untreated film under a scanning electron microscope (Fig. 3A–D). The results were similar to PLA films treated with cultures of *Amycolatopsis* sp. (Pranamuda et al., 1997), *Bordetella petrii* PLA-3 (Jarerat and Tokiwa, 2003), *Saccharothrix waywayandensis* (Tokiwa and Jarerat, 2004) and *Kibdelosporangium aridum* (Kim and Park, 2010), with the surfaces of the PLA films becoming rough, with hemispherical and irregular holes. Using purified PLA depolymerase from *Amycolatopsis* sp. K104-1 also produced the same results (Nakamura et al., 2001).

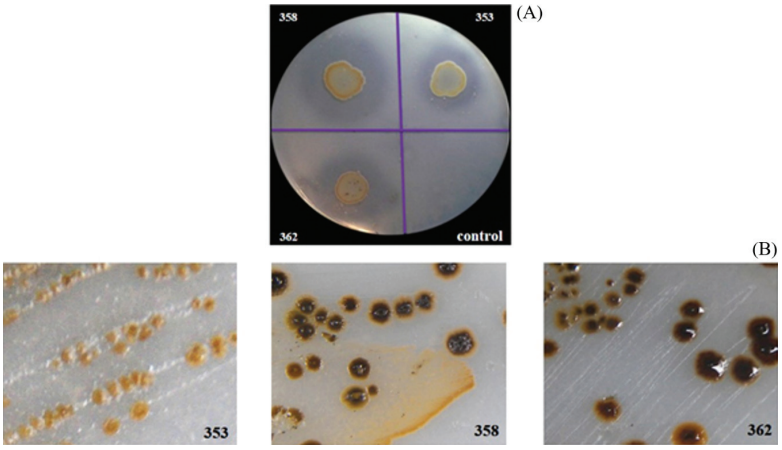


**Fig. 1** PLA-degrading *Micromonospora* spp. and their morphology: (A) clear zone formation of strains GMKU 353, GMKU 358 and GMKU 362 on emulsified PLA agar after incubation at 28°C for 7 d; (B) colony morphology of the three strains on ISP 3 after incubation at 28°C for 2 wk

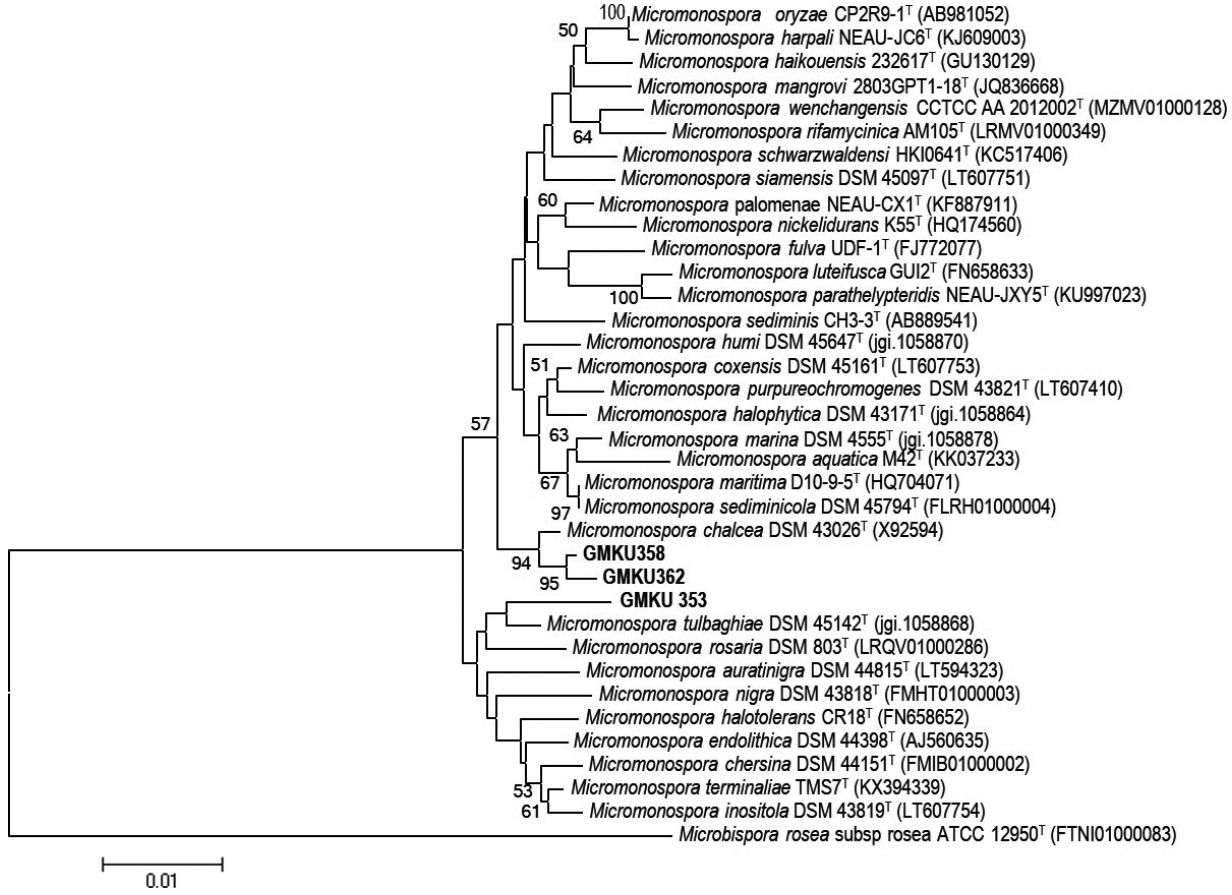


**Fig. 2** Neighbor-joining phylogenetic tree based on16S rRNA gene sequences showing the relationships of strains GMKU 353, GMKU 358 and GMKU 362 with other *Micromonospora* species, where *Microbispora rosea* subsp. *rosea* was used as an outgroup, the scale bar indicates 0.01 substitutes per nucleotide position and bootstrap percentages (1,000 replicates) above 50% are shown at nodes

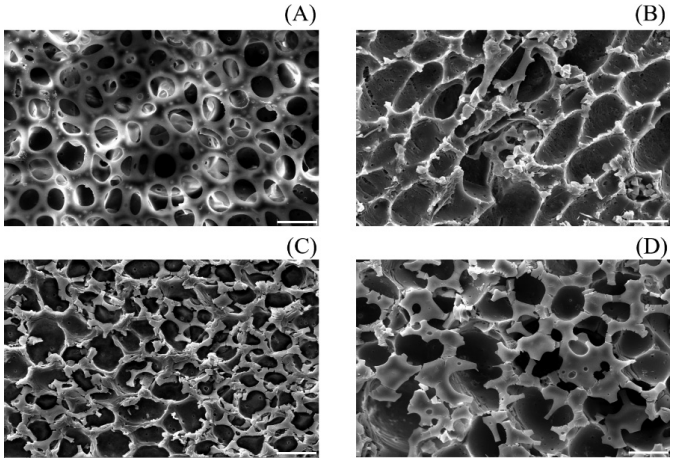


**Fig. 3** Poly (L-lactic acid) film surfaces under scanning electron microscope at 1,000× following incubation at 28°C and 200 revolutions per minute for 2 wk: (A) without inoculation; (B) *Micromonospora* sp. GMKU 353; (C) *Micromonospora* sp. GMKU 358; (D) *Micromonospora* sp. GMKU 362. Bar, 10 μm.

*Surface plasmon resonance analysis*

The PLA-degrading activity of crude enzymes from *Micromonospora* sp. GMKU 353, GMKU 358 and GMKU 362 were determined at 707.58 μg/mL, 711.19 μg/mL and 729.24 μg/mL, respectively. The sharpest slope was determined from the crude enzyme of *Micromonospora* sp. GMKU 358 corresponding to the highest degradation rate (6.16 ng/mm$^2$/min) of the PLA polymer, which was about 2.6 times higher than those of *Micromonospora* sp. GMKU 353 (2.36 ng/mm$^2$/min) and *Micromonospora* sp. GMKU 362 (2.31 ng/mm$^2$/min) (Fig. 4A).

Crude enzyme of *Micromonospora* sp. GMKU 358 was then diluted to 142.20 μg/mL, 71.10 μg/mL, 28.44 μg/mL, 14.22 μg/mL and 7.11 μg/mL to investigate the effect of concentration using SPR. The results indicated that SPR was not able to detect PLA degradation at 7.11 μg/mL of crude enzyme but could detect it at higher concentrations (≥14.22 μg/mL). When 142.20 μg/mL of crude enzyme was used, the specific activity of enzyme degradation rate was highest at 0.02 mg/hr/mg crude enzyme (Fig. 4B). The results suggested that SPR could detect PLA-degrading activity at reasonably low concentrations of crude enzyme that correlated with low degradation rates. The results were in agreement with the α chymotrypsin concentration being directly related to the degradation rate of polymer film (Sumner et al., 2000). However, material properties and environmental factors such as humidity, temperature, pH and the presence of enzymes also affect the degradation of PLA (Nishida and Tokiwa, 1993; Ho et al., 1999; Tsuji, 2010).

The effect of pH on the degradation of PLA was investigated using crude enzyme of *Micromonospora* sp. GMKU 358 at 71.10 μg/mL. The results indicated that the enzyme was active from a neutral to basic pH range of 6–10 and no activity was detected under acidic conditions (Fig. 4C). The optimal degradation rate was 0.22 mg/hr/mg crude enzyme in Tris-HCl pH 10 (Fig. 4C). These current results were similar to previous reports that PLA depolymerases from *Amycolatopsis* sp. K104-1 (Nakamura et al., 2001) and *Laceyella sacchari* LP175 (Hanphakphoom et al., 2013) had optimum activity at pH 9.5. Purified PLAase I, PLAase II and PLAase III from *Amycolatopsis orientalis* sub sp. *orientalis* was reported to have maximum activity at pH 9.5–10.5 (Li et al., 2008). Furthermore, PLA depolymerase from *Actinomadura* sp. T16-1 was reported to have optimum activity at pH 10 (Sukkhum et al., 2009). However, some PLA-degrading enzymes had optimum activity at a neutral pH (6–7), such as *Amycolatopsis* sp. strain 41 (Pranamuda et al., 2001), *Amycolatopsis* sp. strain 3118 (Ikura and Kudo, 1999), *Bacillus licheniformis* (Prema and Palempalli, 2015) and *Aspergillus niger* MTCC 2594 (Nakajima-Kambe et al., 2012).

The effect of ionic strength on PLA degradation by crude enzyme of *Micromonospora* sp. GMKU 358 was investigated using 0.05 M NaCl and 0.25 M NaCl and without NaCl in Tris-HCl at pH 10. The degradation rate decreased as the NaCl concentration increased (Fig. 4D). This result was in contrast with other hydrolases from *Thermobifida fusca*, where a high ionic strength (250 mM NaCl) was necessary to achieve the maximum enzyme activity (Kleeberg et al., 2005; Sinsereekul et al., 2010).

In conclusion, PLA-degrading endophytic *Micromonopora* spp. were successfully obtained using PLA-emulsified agar and PLA film degradation. The activity of crude enzyme was clear based on analysis using SPR. Study is ongoing on the genes related to enzymatic activity from *Micromonospora* sp. GMKU 358.

**Conflict of interest**

There are no conflicts of interest.

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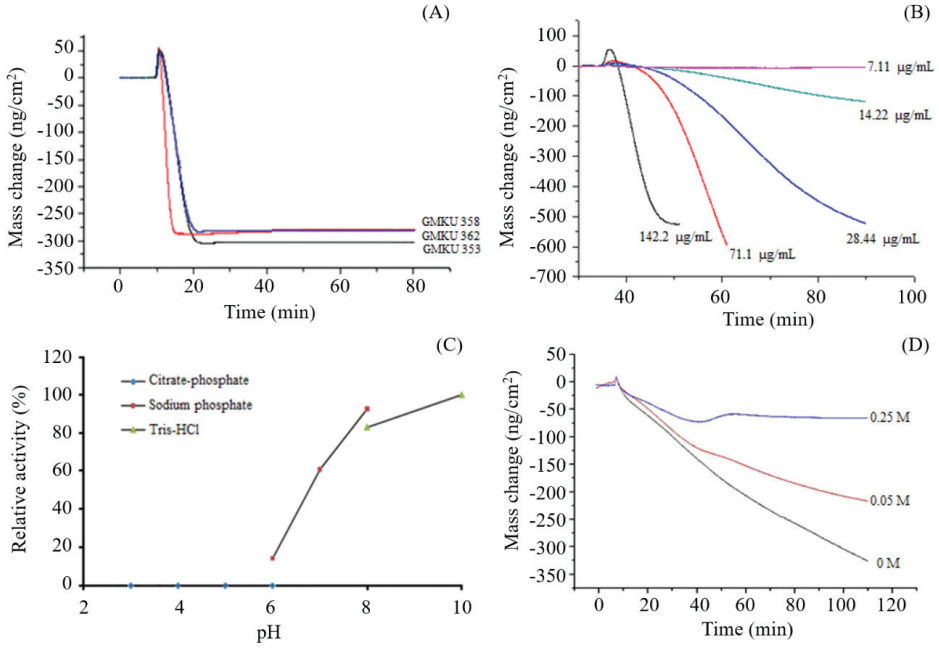


**Fig. 4** Surface plasmon resonance sensograms for degradation of poly (L-lactic acid) (PLA): (A) crude enzymes of *Micromonospora* spp. GMKU 353, GMKU 358, and GMKU 362 at 707.58 µg/mL, 711.19 µg/mL and 729.24 µg/mL, respectively, in sodium phosphate buffer at pH 7.0; (B) crude enzymes of *Micromonospora* sp. GMKU 358 at 142.20 µg/mL, 71.10 µg/mL, 28.44 µg/mL, 14.22 µg/mL and 7.11 µg/mL; (C) effect of pH on degradation of PLA using 71.10 µg/mL crude enzyme of *Micromonospora* sp. GMKU 358 in 0.2 M citrate buffer at pH 3–6, 0.2 M sodium phosphate buffer at pH 6–8 and 0.2 M Tris-HCl at pH 8–10; (D) effect of ionic strength on PLA degradation of 71.10 µg/mL crude enzyme of *Micromonospora* sp. GMKU 358 at 0 M NaCl, 0.05 M NaCl and 0.25 M NaCl in Tris-HCl at pH 10

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