การศึกษากระบวนการรีไซเคิลพลาสติกชีวภาพชนิดกรดพอลีดีแอลแลกติก โดยใช้เอนไซม์ที่ผลิตจากแอคติโนมัยซีทิส

STUDY OF BIOLOGICAL RECYCLING PROCESS OF POLY (DL-LACTIC ACID) USING ACTINOMYCETES ENZYMES

TITIPORN PANYACHANAKUL

GRADUATE SCHOOL Srinakharinwirot University

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STUDY OF BIOLOGICAL RECYCLING PROCESS OF POLY (DL-LACTIC ACID) USING ACTINOMYCETES ENZYMES

TITIPORN PANYACHANAKUL

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STUDY OF BIOLOGICAL RECYCLING PROCESS OF POLY (DL-LACTIC ACID) USING ACTINOMYCETES ENZYMES

BY

TITIPORN PANYACHANAKUL

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Title	STUDY OF BIOLOGICAL RECYCLING PROCESS OF POLY (DL-LACTIC ACID) USING ACTINOMYCETES ENZYMES
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Nowadays, plastics are used to produce many kinds of products which make our lives easier. Since the 1950s, plastic usage has steadily grown every year because of its properties, such as versatility, light weight, flexibility, strong and inexpensive. Meanwhile, plastic pollution has become a serious problem for the environment because it takes many years to degrade. Moreover, it has an effect on wildlife, marine life, and human life, as well as leading to global environmental problems. Therefore, biodegradable plastic an interesting replacement for petrochemical plastic. Poly (lactic acid) or PLA is a biodegradable plastic derived from agricultural products and can be degraded by a variety of enzyme produced by microorganisms. The aim of this study is to complete the biological process consisting of PLA degradation and polymerization using the enzyme produced by Actinomycetes. The production of PDLLA degrading enzyme production by an immobilized *Actinomadura keratinilytica* strain T16-1 on a scrub pad as an immobilizer studied with the maximum enzyme activity of 942.67 U/mL at an aeration rate of 0.25 vvm, an agitation speed of 170 rpm, at a temperature of 45°C for forty eight hours under repeated batch fermentation. Afterwards, the PDLLA degrading enzyme was used for PDLLA degradation by the simultaneous usage of the PDLLA degradation and dialysis method. The results showed the highest percent weight loss in reactions containing PDLLA powder with lactic acid concentration of 18,018 mg/L. Moreover, PDLLA re-polymerization using thermo-solvent tolerant lipase produced by the *Streptomyces* sp. strain A3301 was investigated. The optimum conditions for lipase production by *Streptomyces* sp. strain A3301 was performed in production medium containing 1.5% xylose, 2% yeast extract, 0.01% NH_2HPO_4, pH 7.0 incubated at a temperature of 30°C for three days with the highest enzyme activity of 321 U/mL. Subsequently, PDLLA degraded product (lactic acid) was used as substrate for PDLLA re-polymerization, which was performed using the thermo-solvent tolerant lipase produced by the *Streptomyces* sp. strain A3301 as bio-catalyst. The PDLLA re-polymerization was achieved with a molecular weight of PDLLA product of 577 DA (n=8) under optimized conditions at 10% of freeze dried crude enzyme, at 60°C for eight hours under nitrogen atmosphere. The results indicated that this thesis could offer a solution to the biological recycling of PLA, including the PDLLA degradation and PDLLA polymerization. Therefore, this research might be adopted as a model to develop the technology to reduce the plastic waste in the future.

Keyword : Poly (lactic acid), Actinomadura keratinilytica strain T16-1, PDLLA-degrading enzyme, Fermentation, PDLLA-degradation, Lipase, Streptomyces sp. strain A3301, Optimization, PDLLA polymerization, PDLLA re-polymerization, Bioprocesses, Immobilization

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CHAPTER 1
INTRODUCTION

Plastics provide social, economic and environmental benefits, but their popularity has produced abundant plastic wastes. Disposing of plastic wastes is a serious problem because plastics barely decompose in the environment. Biodegradable polymers such as poly (lactic acid) or PLA, poly-β-hydroxybutyrate (PHB) and polycaprolactone (PCL) have been developed as replacements for petrochemical plastic materials. Poly (DL-lactic acid) or PLA is an aliphatic polyester produced from lactic acid monomers. It has been used in several purpose including food packaging, automotive interior parts, agriculture geotextiles, furniture, apparel and nonwovens ([1, 2]). Disposal methods for PLA have been investigated, such as composting and recycling. To date, many reports have demonstrated the ability of various microorganisms to degrade PLA such as *Bacillus brevis*, *B. stearothermophilus*, *B. licheniformis*, *B. amyloliliquefaciens*, *Geobacillus thermocatenulatus*, *Amycolatopsis thailandensis*, *Cladosporium*, *Purpureocillium*, *Laceyella sacchari*, *Thermoactinomyces vulgaris*, *Actinomadura keratinilytica*, *Nonomuraea* strains, *Bordetella petrii* PLA-3, *Stenotrophomonas maltophilia* LB2-3, *Thermopolyspora flexuosa, Pseudonocardia* sp. RM423, *Stenotrophomonas pavanii* and *Pseudomonas geniculate* ([3-11]). However, the enzyme production by these reported strains and its scaling up have not been studied so far. This study focuses on our group previous Actinomycetes that is *Actinomadura keratinilytica* strain T16-1. The strain T16-1 presented a high ability to produce PDLLA-degrading enzymes with 257 U/ml after incubation for 3 days under optimal conditions with aeration rate of 0.43 vvm, pH of 6.85 and temperature at 45°C in a 3-L airlift fermenter ([10]). Moreover, the crude PDLLA -degrading enzyme could be adsorbed to the PDLLA powder used as substrate and converted the PDLLA powder to produce lactic acid as degradation products. Therefore, the main application of the PDLLA-degrading

enzyme produced by strain T16-1 was biological recycling, as previously reported ([12]). Currently, enzymes that degrade PLA, such as lipases and proteases, are being used for many purposes. A second strategy for the application of PLA-degrading enzyme was reported. The enzyme was mixed with biopolymer film containing PLA to control the degradation when used in the agricultural field ([13]). 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 ([6, 14]). 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 ([15-17]). However, in each fermentation process, free cells were not suitable due to the possibility of substrate or product inhibition through direct contact between the cells and medium ([18]). To resolve these problems, a cell immobilization method was applied for the fermentation processes. Cell immobilization can help to decrease the cost of fermentation and has many advantages such as achieving higher cell concentration resulting in a higher fermentation rate, yield and productivity as well as recycling cell utilization with reduced cost and time ([19]).

In addition, PLA is generally polymerized via a chemical process as poly condensation from lactic acid and ring opening polymerization from lactide ([20, 21]). Nevertheless, the chemical processes require the extreme conditions, such as high temperature, high concentration of organic solvent and high adding of catalysts. Whereas, the biological polymerization processes showed an alternative process that requires mild conditions and that uses an enzymatic method ([13]) such as the lipase was applied for PLA polymerization as a bio-catalyst. The lipase was used for modification of PLA through bioprocessing, that replace the chemical process. Moreover, in the PLA re-polymerization by bioprocess requires organic solvent such as toluene and hexane for PLA synthesis at temperature 50-70°C. Therefore, the lipases were used in re-polymerization should have ability for organic solvent tolerant and thermo-stable. The previous reports about biological re-polymerization of PLA. In 2008, Lassalle and

Ferreira reported the optimal conditions for biological re-polymerization were performed at temperature 65°C for 96 hours by lipase under hexane condition ([20]). Chuensangjun et al. reported the low molecular weight of PLA is drawn from the commercial lactic acid by the commercial lipase, Lipozyme TL IM at optimum condition temperature 50°C for 5 hours ([21]). Recently, in 2016 Youngpreda et al. investigated the PDLLA degradation by PDLLA -degrading enzyme produced from *Actinomadura keratinilytica* strain T16-1 at optimum condition as temperature 60°C for 24 hours released product with 6,843 mg/L of lactic acid. Subsequently, that degradable products were used as substrate for re-polymerized of PDLLA by the commercial lipase as catalyst under nitrogen atmosphere for 6 hours. A re-polymerized PLA oligomer was completed with a molecular weight of 378 Da (n=5) ([22]).

Interestingly, the PDLLA degradation conditions and the recycling process have not been investigated so far. Afterward, recycling of biopolymers, such as PDLLA, which uses microbial enzymes, should be described in more detail, especially the processes of biodegradation, bio-recycling and re-polymerization to develop new technologies for reducing plastic waste in the future.

This work, we focused on the biological process of PDLLA degradation and polymerization process as shown in Figure 1 ([23]). Therefore, the optimization of PDLLA-degrading enzyme production and degradation were determined. Moreover, thermo-solvent tolerant lipase produced by isolated actinomycete was subjected to study the enzyme purification and characterization for re-polymerization of PDLLA. This thesis was used as a model to complete the biological recycling of PDLLA including degradation and polymerization.

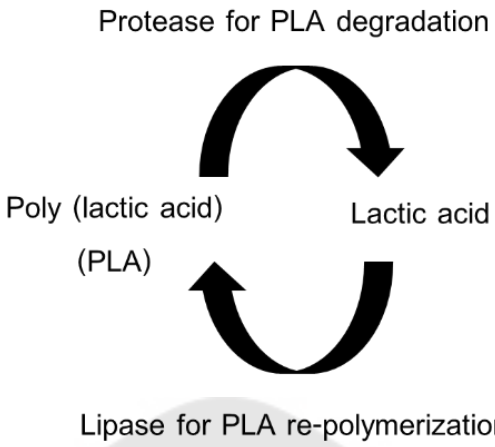


FIGURE 1 The scheme of PLA degradation and synthesis by biological method.

Objectives of the study

1. To optimize for PDLLA degrading enzyme production under 5-L and 10-L stirrer fermenter using the immobilized *Actinomadura keratinilytica* strain T16-1.

2. To scale up and optimize for PDLLA degradation condition by the enzyme produced by *Actinomadura keratinilytica* strain T16-1.

3. To screen and identify the thermo solvent tolerant lipase producing actinomycete.

4. To purify and characterize the thermo solvent tolerant lipase produced by isolated actinomycete.

5. To evaluate the re-polymerization process of PLA by using lipase produced by isolated actinomycete.

Scope of the study

Optimization of PDLLA-degrading enzyme production by immobilized *Actinomadura keratinilytica* strain T16-1 in 5-L stirrer bioreactor under various fermentation process including batch, repeated batch and continuous fermentation were investigated. Afterwards, scale up of PDLLA-degrading enzyme production to 10-L stirrer bioreactor was studied. After that, optimization of PDLLA-degradation condition was determined using the enzyme produced by *Actinomadura keratinilytica* strain T16-1. To complete biological process for PDLLA polymerization, screening of thermo solvent tolerant lipase producing actinomycetes was investigated. The crude enzyme produced by selected actinomycete strain was subjected to re-polymerize PDLLA.

CHAPTER 2
LITERATURE REVIEW

1. Biodegradable polymers

1.1 Definitions of biodegradable polymers and bio-plastics

Several definitions of biodegradable polymers were defined depending on the application of polymer.

"CEN (1993) - A degradable material in which the degradation results from the action of microorganisms and ultimate materials are converted to water, carbon dioxide and/or methane and a new cell biomass."

"BPS Japan (1994) - Biodegradable plastics are polymeric materials which are changed into the lower molecular weight compounds where at least one step in the degradation process is through metabolism in the presence of naturally occurring organism."

"Japan Bio-plastics Association (JBPA) (1999) - Biomass-based plastics as polymer materials that are produced by synthesizing, either chemically or biologically, materials which contain renewable organic materials (natural organic polymer materials that are not chemically modified are excluded)."

"Ray and Bousmina (2005) - Biodegradable polymers are defined as those undergo microbially induced chain scission leading to the mineralization ([24])."

"Nair and Laurencin (2007) - Biodegradable polymers are polymer from both synthetic polymers and biologically derived (or naturally) polymers have been extensively investigated as biodegradable polymeric biomaterials ([25])."

"Boards, Pollet and Avérous L (2009) - Biodegradable polymers are polymers from both of chemically synthesized or biosynthesized during the organism grows, that are degraded by some microorganisms or enzymes ([26])."

"Ulery, Nair and Laurencin CT. (2011) - A biomaterial is defined as any natural or synthetic substance engineered to interact with biological systems in order to direct medical treatment ([27])."

"Pullman et al (2013) – Bio-plastics can be defined as plastics based on renewable resources or as plastics which are biodegradable or compostable" ([28]).

1.2 Classifications of biodegradable polymers

Biodegradable polymers are sorted into four different categories, depended on the synthesis method ([29]) as shown in Figure 2 ([29]). The biodegradable polymers can be divided into two main families, that are the agro-polymers or natural polymers (categories A) and the synthetic biomaterials also called bio-polyesters (categories B-D). The structure of biodegradable polymers as shown in Table 1

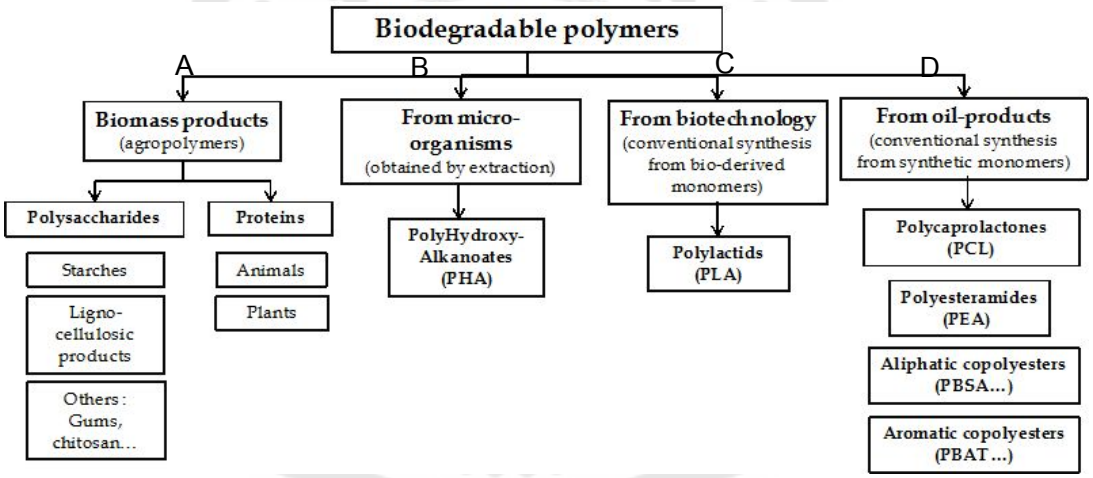
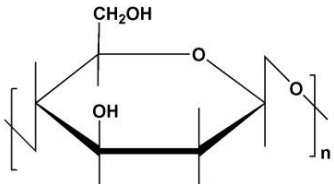
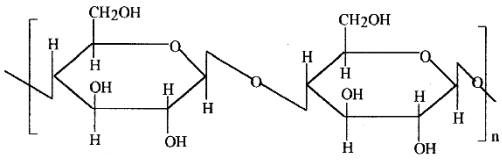
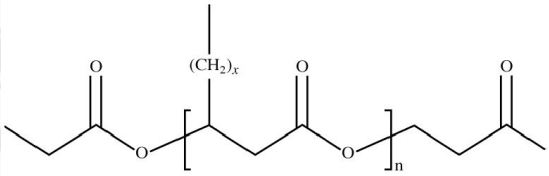
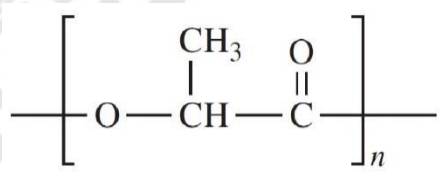
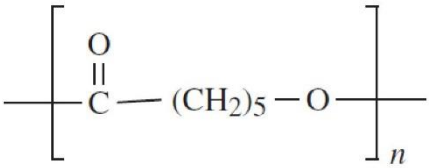


FIGURE 2 Classification of biodegradable polymers

(A.) Polymers from biomass, (B.) Microbial polymers, (C.) Polymers from chemically synthesized with agro-resources monomer and (D.) Polymers which monomers and polymers are produced by chemical synthesis with fossil resources

TABLE 1 The structure of biodegradable polymers

Type of biodegradable polymers	Structures
1. Biodegradable polymers from biomass	
Chitosan ([30])	
Cellulose ([31])	
2. Polymer is derived from microorganism	
Polyhydroxyalkanoates (PHA) ([31])	
3. Polymers from biotechnology	
Poly (Lactic acid) (PLA) ([2])	
4. Polymers from petrochemical product	
Polycaprolactone (PCL) ([31])	

1.3 Poly (Lactic acid) (PLA)

Poly (Lactic acid) or PLA is synthesized from lactic acid monomers, which are derived from the agricultural resources such as corn, cassava, sugarcane and rice in virtue through fermentation by microorganisms as shown in Figure 3 ([32-36]).

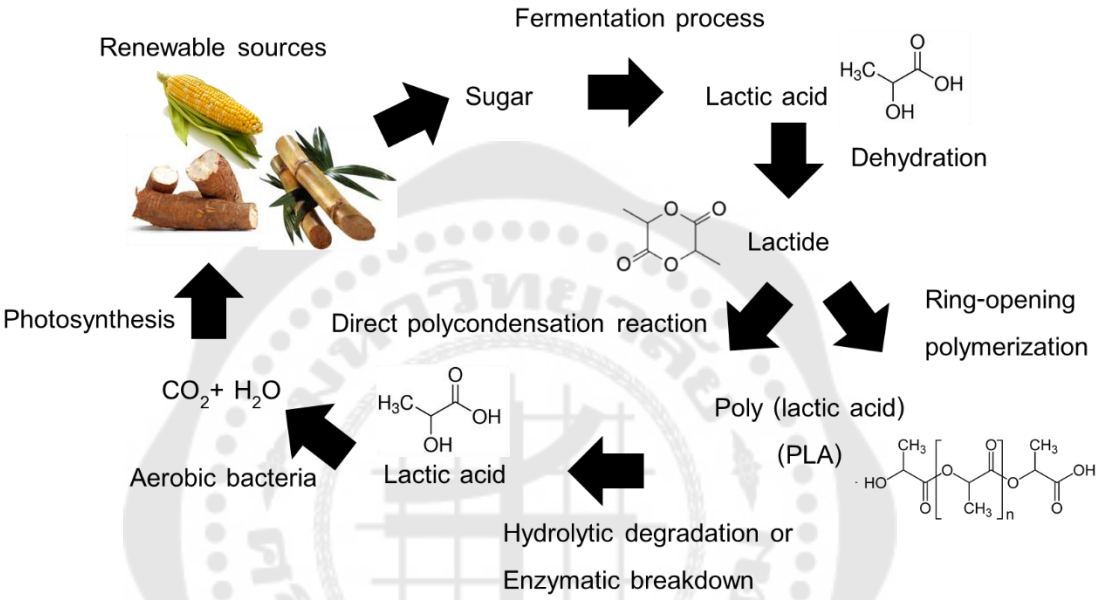


FIGURE 3 PLA synthesis from renewable agricultural resources by combination of biological and chemical polymerization processes

Lactic acid is the basic monomer for PLA synthesis. It is chemically known as 2-hydroxy-propanoic acid ($HOCH_3CHCOOH$) . The lactic acid is existent two stereoisomers, which are L- and D-lactic acid which can be delivered from different ways as shown in Figure 4 ([37]). Most the lactic acid is naturally formed as L-lactic acid and reached to poly (L-lactic acid) or PLLA with low molecular weight through polycondensation. On the other hand, a racemic mixture of L-lactic acid and D-lactic acid can be made through the chemical synthesis. Certainly, lactic acid was synthesized to reach to the cyclic dimer form by chemical reaction ([38]). Both lactic acid forms can be created to lactide with three stereo forms. There are L-lactide, D-lactide

and meso-lactide which is the mediate step of PLA synthesis as shown in Figure 5 ([38]). The physical properties of lactic acid is shown in Table 2 ([38]).

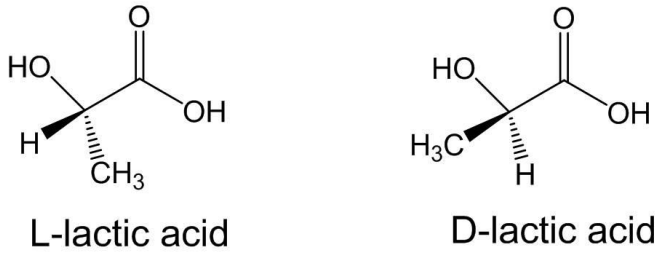


FIGURE 4 Stereoisomers of lactic acid

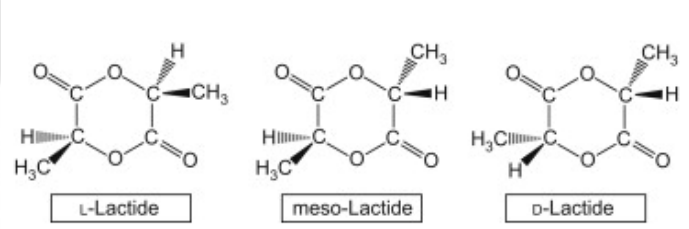


FIGURE 5 Lactide stereocomplex

TABLE 2 Physical properties of lactic acid.

Parameters	Properties
Chemical formula	$C_3H_6O_3$
Chemical name (IUPAC)	2-hydroxy-propanoic acid
Molecular weight	90.08
Physical appearance	Aqueous solution
Taste	Mildly sour
Melting point	53˚C
Boiling point	>200˚C
Solubility in water (g/100 g H_2O)	Miscible
Dissociation constant (Ka)	1.38 X 10^{-4}
pKa	3.86
pH (0.1% solution, 25˚C)	2.9

1.4 Applications of Poly (lactic acid)

Poly (lactic acid) or PLA is a biodegradable polymer. The important properties of PLA are a strength, non-toxic and biodegradable. PLA has many applications including household, engineering and agricultural, medical, pharmaceutical and industry.

1.4.1 Poly (lactic acid) for domestic applications

PLA for domestic such as apparel, it has excellent wicking properties, low moisture, smell keeping and no skin irritation. Bottle, PLA bottle is suitable, glossy and inflexible at high temperature (50-60˚C). Home textile, PLA can be transformed into a fiber, which is a good ventilate, luxury, quick drying, smooth and easy to tend ([38]).

1.4.2 Poly (lactic acid) for engineering and agricultural applications

PLA is appropriated for engineering applications, mostly using PLA in secondary applications and electrical or electronic applications such as engineering material, automotive, building materials ([38]).

In an agricultural application using PLA for soil protection, weed-killer and compost retention. PLA mulch film, is slow degradable and ultimately decomposed when the crops are harvested ([38]).

1.4.3 Poly (lactic acid) for medical applications.

PLA widely uses in scaffolds to provide impermanent support for attachment and rehabilitate of tissues during surgery. In pharmaceutical it is being used as a drug carrier for control drug release system ([38]).

1.4.4 Poly (lactic acid) for industrial applications.

PLA is a compostable polymer so it is used in many industries, especially in food industry such as cups and food service ware, this application can be used to reduce non-degradable plastics. PLA is a good alternative raw material because it has transparency, inflexibility, good barrier with oil and moisture. Films for packaging bag of a bakery, salad, candy and foam trays are used for fresh food packaging, which can fully be degraded in a short period of time and furnish nutrient in soil with compost ([38]).

2. PLA degradation

PLA can be degraded by a biological and chemical mechanism. PLA is unable to be directly broken down and consumed by living cells as effectively as lactic acid itself. The main factors have an effect on PLA biodegradation are stereochemistry, crystallinity and molecular weight of PLA. Study on PLA had shown that the crystalline PLA is more resistant to degradation than the amorphous PLA ([39-42]). The PLA has an equal balance between amorphous regions and crystalline regions which is better for hydrolysis than only amorphous regions of PLA ([40]). Researchers had observed the high-molecular-weight polyesters which are degraded at a slower rate ([43]).

The biological process includes microbial and enzymatic activities. They are important for PLA degradation. The degradation of PLA implicates PLA-depolymerase or PLA-degrading enzyme and other proteases produced by PLA-degrading microorganisms ([44]).

2.1 Microbial degradation of PLA

Different environment have many diversities of polymer-degrading microorganisms as shown in Table 3. Therefore, studying the ecological and taxonomic of polymer-degrading microorganisms are highly important because they are responsible for the degradation of plastic materials. Polymers are degraded in the soil by the variety of microorganisms. The favorite methods for assessment of the polymer-degrading microorganism population in the environment are the plate count and clear zone methods. However, the PLA-degrading microorganisms are not vastly distributed in the natural environment so PLA is less sensible to microbial attack in the natural environment than other microbes and synthetic aliphatic polyesters ([44]). Molecular weight oligomers and then mineralized into CO_2 and H_2O. Nevertheless, The PLA can be degraded in soil by the burial method, but that is slow and take a long time to begin the degradation ([44]). Table 3 showed PLA-degrading microorganisms which is the first report of PLA degradation by proteinase K produced from *Tritirachium album* demonstrating a broad spectrum as serine protease ([45]). The research of PLA degradation by actinomycetes had been reported. *Amycolatopsis* strain HT-32 showed the ability to form clear zone on PLA agar. After this finding, many researchers were focused on PLA degradation by microorganisms ([46]). The first isolated PLA degrading microorganism was *Amycolatopsis* strain HT-32, which could degraded 60 mg of PLA film after 14 days in liquid medium ([46]). In addition, 15 strains of PLA-degrading actinomycestes of genus *Amycolatopsis* sp. was investigated by using clear zone around colony on emulsified PLA agar plate method, which indicated that a large distribution of PLA degraders within this genus. In another study, most of PLA-degrading microorganisms were reported as a genus *Amycolatopsis* ([47-49]). Isolation of PLA-degrading microorganisms led to the isolation of 4 actinomycetes and 4 bacteria; one of actinomycetes was classified as a member of genus *Amycolatopsis* based on 16S rDNA sequencing. The report of PLA degrading actinomycete was taxonomically similar *Amycolatopsis* sp. and isolated from soil supernatant, pond water or river on solid plate by spread method ([47]). The Isolate *Amycolatopsis* used silk as inducer for confirming the

degradation ability to form clear zone on PLA plate ([48]). Isolation of *Amycolatopsis* sp. strain K104-1 and K104-2 from 300 soil samples were shown in the PLA degrading activity ([49]). Several studies on PLA degrading microorganisms were reported. In 2003, a new PLA degrading actinomycetes was *Kibdelosporangium aribum* which can degraded more than 97 mg out of 100 mg of high molecular weight PLA film (Mn: 3.4 x 10^5) within 14 days in liquid culture. L-lactic acid, the monomeric degradation product of PLA was totally assimilated by the strain and in solid culture, many distinct grooves formed by the morphology of filamentous microorganisms on the surface of PLA film were observed by scanning electron microscopy (SEM) ([50]). Moreover, PLA was degraded by more than 95 mg from 100 mg PLA film by *Saccharothrix waywayandensis* in liquid medium containing 0.1% (w/v) gelatin ([50]). PLA degrading fungus, *Tritirachium album* and two strains of Actinomycetes, *Lentzea waywayandensis* and *Amycolatopsis orientalis* were demonstrated in the use of some protein (poly-L-amino acid), peptides and amino acid as enzyme inducer. The enzyme activity produced variety between different strains and different inducers. *Amycolatopsis orientalis* used silk fibroin as the best inducer. While, *Tritirachium album* and *Lentzea waywayandensis* used elastin as the best inducer ([50]). In 1999, The first thermophilic PLA-degrading bacteria, *Brevibacillus* sp. (formerly *Bacillus brevis*) was reported ([3]). Thermophilic L-PLA-degrading bacteria from garbage fermenter, was *Bacillus smithii*. This strain grew well in the medium containing 1% L-PLA and reduced molecular weight of L-PLA by 35.6% after 3 days of incubation at 60°C under shaking condition ([51]). The newly thermophilic PLA film degrading microorganisms from 153 soil samples by enrichment culture technique at optimum growth temperature of around 60°C for 20 days. The strain has high specific of PLA and without esterase activity in culture broth. This strain was identified as nearly as *Geobacillus thermocatenulatus* ([4]). Lately, newly thermophilic bacteria from compost was identified as *Bacillus licheniformis,* which had ability to degrade higher molecular weight of PLLAs at 58°C ([52]). Further study, in 2009, the report talked about the isolation of a novel poly (L-lactic acid) degrading actinomycetes from Thai forest soil, was *Actinomadura keratinilytica* strain T16-1. It formed clear zone on an

emulsified PLA agar plate and produced the highest PLA-degrading activity in liquid medium containing PLA film as a carbon source ([10]). In 2011, a novel actinomycete isolated from soil sample in northern Thailand, *Amycolatopsis thailandensis* strain CMU-PLA07^{T} has ability to degrade PLA ([53]). In 2014, the PLA- degrading thermophilic bacteria from forest soils was isolated, they have 11 isolates forming clear zone on emulsified PLA agar plate at 50˚C. Among the isolates, strain LP175 showed the highest PLLA degrading ability. It was closely related to *Laceyella sacchari*, with 99.9% similarity based on the 16S rRNA gene sequence ([6]). The biodegradability of PLA by the actinomycete *Pseudonocardia* sp. RM423, which had ability to degrade PLA for 4 weeks of cultivation in basal salt medium broth (BSM) with 0.3% (w/v) gelatin as an enzyme inducer ([54]). In addition, The PLA-degrading microorganism that *Stenotrophomonas maltophilia* LB2-3 was isolated by using clear zone method on agar plate containing 0.1% PLA after incubation at 37°C for 7 days ([55]). The extracellular PLA-degrading enzyme produced from *Amycolatopsis orientalis* showed potent degrading activity, which is effective for biological recycling of PLA, 2,000 mg/L of PLA powder was completely degraded within 8 hours at 40°C using 20 mg/l purified enzyme ([56]).

TABLE 3 PLA-degrading microorganisms, type of enzyme and detection method for PLA-degradation test.

Microorganism	Type of enzyme	Detection method of PLA degradation	Reference
Amycolatopsis sp. HT32	Protease	Film-weight loss	([46])
Amycolatopsis sp. 3118	Protease	Film-weight loss	([47])
Amycolatopsis sp. KT-s-9	Protease	Clean zone method	([48])
Brevibacillus	Protease	Change in molecular production and viscosity	([3])
Amycolatopsis sp. 41	Protease	Film-weight loss	([46])
Amycolatopsis sp. K104-1	Protease	Turbidity method	([49])
Bacillus smithii PL 21	Lipase	Change in molecular production and viscosity	([51])
Tritirachium album ATCC 22563	Protease	Film-weight loss	([57])
Lentzea waywayandensis	Protease	Film-weight loss	([50])
Kibdelosporangium aridum	Protease	Film-weight loss	([50])
Bacillus stearothermophilus	Protease	Change in molecular production and viscosity	([5])
Paenibacillus amylolyticus TB-13	Lipase	Turbidity method	([58])
Geobacillus thermocatenulatus	Prorease	Change in molecular production and viscosity	([4])
Cryptococcus sp. S-2	Lipase	Turbidity method	([59])

TABLE 3 (Continued)

Microorganism	Type of enzyme	Detection method of PLA degradation	Reference
Bacillus licheniformis PLLA-2	-	Biodegradation test	([52])
Bacillus clausii pLA-M4	Lipase	Molecular technique	([60])
Bacillus cereus pLA-M7	Lipase	Molecular technique	([60])
Treponema denticola pLA-M9	Hydrolase	Molecular technique	([60])
Paecilomyces	-	Molecular technique	([61])
Thermomonospora	-	Molecular technique	([61])
Thermopolyspora	-	Molecular technique	([61])
Actinomadura keratinilytica T16-1	Serine protease	Clear zone method and turbidity method	([10])
Micromonospora echinospora B12-1	Serine protease	Clear zone method and turbidity method	([10])
Micromonospora viridifaciens B7-3	Serine protease	Clear zone method and turbidity method	([10])
Nonomuraea terrinata L44-1	Serine protease	Clear zone method and turbidity method	([10])
Nonomuraea fastidiosa T9-1	Serine protease	Clear zone method and turbidity method	([10])
Laceyella sacchari LP175	Serine protease	Clear zone method and turbidity method	([6])
Stenotrophomonas maltophilia LB2-3	-	Clear zone method	([11])
Stenotrophomonas pavanii CH1	Protease	Clear zone method	([11])
Pseudomonas geniculate WS3	Protease	Clear zone method	([11])

2.2 Enzymatic degradation of PLA

Enzymes play a key role in the polymer degradation. Microbial protease and lipase were reported to present the capability to degrade PLA. Protease can degrade PLA by recognition at α-ester bond as same as α-ester bond of peptide. Lipase can degrade PLA because of lipase constitutes a valuable group of esterase for aliphatic polyesters degradation. The mechanism of lipase, which claves the ester bond randomly parallel with main chain of polymer substate ([13, 62]). Nevertheless, the lipases could not cleave the PLA indicated that lipase can degrade different aliphatic polymers which had a low T_m, no chiral carbon atom, a relatively large number of methylene groups between ester and ester bonds and likewise amorphous polymer. Conversely, PLA degrading enzymes selectively cleaved the **α**-ester bond of L-isomer. Commonly, occurring amino acids as constituents of protein are L-isomer. The proteinase K could not cleave the D-stereoisomer of PLA. It is reasonable to determine that PLA degrading enzyme is protease-type enzyme that recognizes the repeated L-lactic acid unit of PLA like L-alanine unit of silk fibroin (protein) ([63]).

The mechanism of aliphatic polyesters degradation by the enzymatic, which is hydrolysis with the two-step process. The first step is enzyme adsorption on the substrate surface through the surface-binding domain and the second step is hydrolysis of the ester bond. The mechanism of PLA-degrading enzyme production and degradation presence of inducers, the production of microbial depolymerase (PLA-degrading enzyme and another protease) is induced. PLA-degrading enzyme can degrade both PLA and protein substrates, but protease degrades only the protein substrates. Finally, the degradation products of proteins by both enzymes, e.g., amino acids and peptides, induced the production of microbial depolymerase enzymes as shown in Figure 6 ([50]).

The first report about PLA- degrading enzyme was proteinase K produced from *T. album* ([46]). Afterwards, this enzyme has been used in many studies on degradation mechanisms of L-PLA ([64]), co-polymers of PLA ([65]) and PLA blends ([66]). In a number of reports that were studied, enzymatic degradation of the amorphous and

crystal structure of PLA has been degraded by using proteinase K, the result proposes that the enzymatic hydrolysis in the amorphous region is faster reaction than the crystalline region ([42, 65, 67, 68]). The PLA-degrading enzyme produced from *Amycolatopsis* strain-41 had higher substrate specificity on PLA than proteinase K ([46]). In addition to the PLA-degrading enzyme activity could be improved by increasing proteinous substance such as gelatin, silk, fibroin, elastin, peptide and amino acids, which indicates that proteases-type enzymes have been produced ([44]). The tree novel PLA-degrading enzyme, named PLAase I, II and III produced from *Amycolatopsis orientalis* ssp. orientalis were identified as a serine-like protease ([69]). The PLA-degrading enzyme produced by *Actinomadura keratinilytica* strain T16-1 had the highest PLA-degrading activity in the liquid culture using PLA film as a carbon source. The PLA-degrading enzyme was purified to homogeneity shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was 30.0 kDa. The optimal pH and temperature were 10.0 and 70ºC, respectively and should be classified as serine protease ([10]). *Laceyella sacchari* strain LP175 produced PLA-degrading enzyme that was classified as serine protease ([6]). Moreover, *Amycolatopsis* sp. strain SCM_MK2-4 showed the highest enzyme activity for both PLA and PCL, 0.046 and 0.023 U/mL, respectively. The enzyme of this strain were protease, esterase and lipase which were able to degrade the PLA film of 36.7% after 7 days of cultivation at 30ºC in culture broth ([8]). Furthermore, PLA degradation by the commercially proteases was measured the product as lactic acid after the end of reaction by boiling for 5 minutes. Most of PLA degrading enzyme was classified as alkaline proteases ([70]). However, their reports showed that subtilisin as a serine alkaline protease, did not degrade PLA under the same condition. On the other hand, the subtilisin showed L-PLA-degradation ability ([44]) and which similar to the purified α-chymotrypsin ([71]). Furthermore, in 2018, PLA-degrading bacteria were isolated on emulsified PLA agar. Among, two strains was identified as *Stenotrophomonas pavanii* CH1 and *Pseudomonas geniculate* strain WS3 that produced PLA-degrading enzyme as protease by using gelatin as inducer and determination of PLA degradation by PLA-weight loss method ([11]).

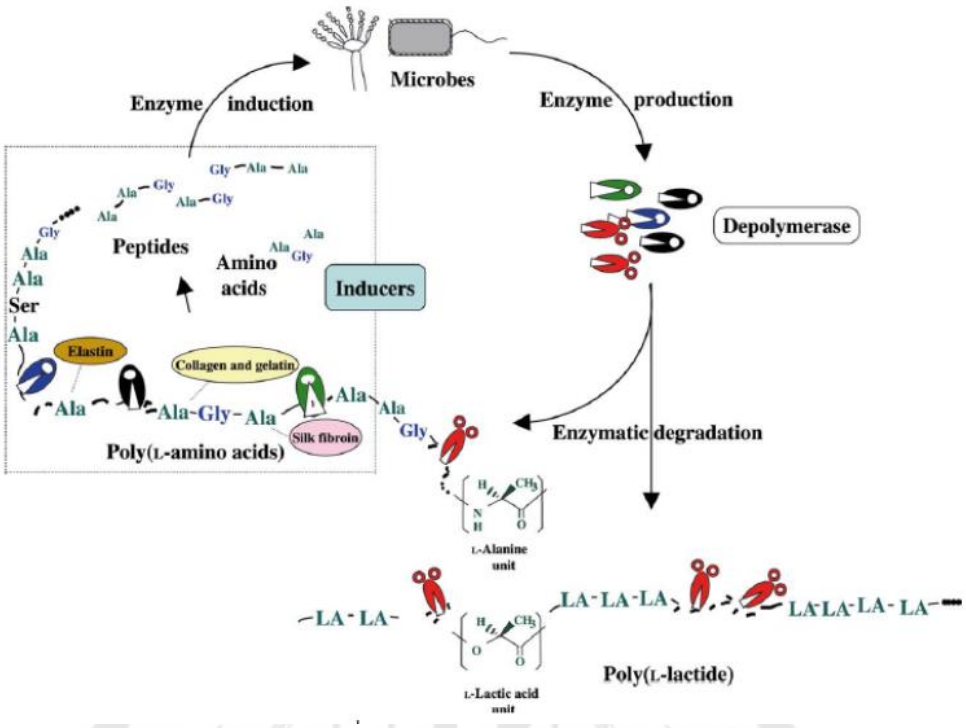


FIGURE 6 Scheme of induction for production and enzymatic degradation of a PLA-degrading enzyme.

2.3 PLA-degrading enzyme production

2.3.1 Microbial Cell immobilization

The study of fermentation process development is highly important for maximizing enzyme production. 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 ([15, 17, 19]). However, in each fermentation process, free cells were not suitable due to the possibility of substrate or product inhibition through direct contact between the cells and medium ([18]). These problems resolve by using a cell immobilization method for the fermentation processes. Cell immobilization can help to decrease the cost of fermentation and has many advantages such as achieving higher cell concentration resulting in a higher fermentation rate, yield and productivity as well as recycling cell utilization with reduced cost and time ([19]).The immobilization of microbial cells in a biological process is performed by a natural

incident or manufacture process. The microbial cells are being used in an industrial biotechnology process, which are applied in the fermentation process. The classical fermentation process has many limitations, for instance; low density of cells, nutritional restrictions and a long downtime of batch-mode performance. The density of the microbial cell is an important factor to increase volumetric productivity. The improvement process to increase the cell populations within fermenter, which use the continuous fermentation with a free cell or immobilized cell. The free cell system cannot perform under a chemostatic condition that decouples specific growth rate and dilution rate. Therefore, the immobilization technology is interested in the cell population control in a fermenter. The advantages of this technology are the liberation to decide the cell density prior to fermentation, the simplify performance of microbial fermentation under continuous process without cell washout, the whole immobilized cell is easier separate from the cellular synthesis of favored compounds, the increase of the microbial cell concentration is higher for the fermentation rate, and this can be applied to recycling utilization of microbial cell and prevented from product inhibition.

The cell immobilization process is the attachment of the whole cells or their permission with the solid phase that allow an exchange of substrates, products, and inhibitors. However, separating the catalytic cell biomass from volumetric phase includes substrates and products. The immobilization method of a cell has various techniques such as adsorption, covalent bonding, cross-linking and entrapment ([72]) as shown in Table 4. The properties of supporters for cell or enzyme immobilization such as low dissolution, difficult to degrade, high durability, high diffusion, low cell explosion, cheap and easy for immobilization as shown in Table 5 ([73]). Many previous reports present the immobilization method being applied for enzyme production such as acrylamide was used as carrier for *Bacillus megaterium* immobilization for produced β-amylase by cross-linking method ([74]). Moreover, Glucoamylase was produced by immobilized *Aureobasidium pullulans* on Ca-alginate ([75, 76]). *Bacillus pumilis* was immobilized by adsorption on scotch brite for xylanase production ([77]). Summary of microbial enzyme production by various immobilization method were shown in Table 6.

2.3.1.1 The methods of cell immobilization

- Adsorption

Adsorption is the first method for cell immobilization. The adsorption is accidental ground on electrostatic interactions such as van der walls forces between the charge of supporter and microorganism cell surface, that presents potential on both plays an important role in cell-support interactions ([72]). The supporter uses adsorption method such as wood, glass ceramic, plastic material, an ion exchange resin ([72]), unpeeled sweet sorghum stalk ([19]), corn stalk bagasse ([78]) and DEAE-cellulose ([79]).

- Covalent bonding

Covalent bonding method has a mechanism based on covalent bond construction between activated inorganic support and cell in the presence of a binding agent. The advantages of this method is the stable nature of the bond between microbial cells and matrix. The supporter uses a covalent bonding method such as silica bead ([72]).

- Cross-linking

Microbial cells can be immobilized by cross-linking by connate between microbial cells and supporter with a chemical or physiological method. The chemical process using glutaraldehyde or toluene diisocyanate as a reagent to cross-link and in the physical method using flocculation and palletization with a polyamine, polyethyleneimine and polystyrene sulfonate. This method has benefits including cheap and strong mutual adherence forces of some microbial cell culture. The supporter uses cross-linking method such as alkylamine glass ([72]).

- Entrapment

The most wildly studies method in cell immobilization is the entrapment of microbial cells in polymer matrixes. The entrapment methods are based on the admittance of cells inside a rigid network to prevent the cells from diffusion into the medium while still allowing penetration of the substrate. The supporter uses entrapment method such as agar, alginate, k-carrageenan, cellulose, collagen, gelatin, polyacrylamide ([72]).

TABLE 4 Summary of immobilization method.

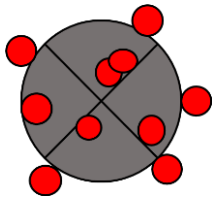
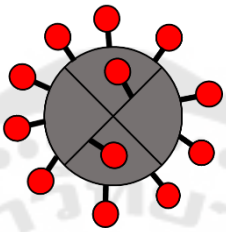
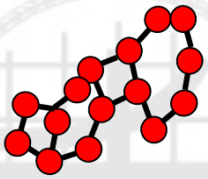
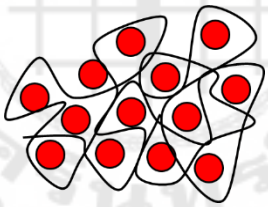
Immobilization method	Structures	Advantage	Disadvantage
1. Adsorption		Cheap, Mild, Reusable, simple	Cell leakage, Sensitive to pH, changes
2. Covalent binding		Permanent	Toxicity, Expensive
3. Cross linking		Mild, Reusable, Simple,	Diffusional limitations, Toxicity, Expensive
4. Entrapment		Mild, Simple	Diffusional limitations, Toxicity, Expensive

TABLE 5 The important properties of supporters for cell/enzyme immobilization.

Properties	Criterion
1. Dissolution	Low
2. Biodegradation	Low
3. Durability	High
4. Diffusion	High
5. Immobilization method	Simple for cell growth
6. Cell explosion	Low
7. Cost	Low

TABLE 6 Summary of microbial enzyme production using various immobilization method.

Type of enzymes	Microorganisms	Supporters	Immobilization methods	References
β-amylase	*Bacillus megaterium*	Acrylamide	Cross-linking	([74])
Glucoamylase	*Aureobasidium pullulans*	Ca-alginate	Entrapment	([75, 76])
Protease	*Humicola lutea*	Wort agar	Entrapment	([80])
Chitinase	*Micromonospora chalcae*	Ca-alginate	Entrapment	([81])
Pectinase	*Aspergillus niger*	Wheat	Adsorption	([82])
Lipase	*Sporotrichum thermophile*	Ca-alginate	Entrapment	([55])
	Rhizopus oryzae	Polyurethane foam	Entrapment	([83])
Lignin peroxidase	*Phanerochaete chysogenum*	Polystyrene-divinylbenzene	Entrapment	([84])
Xylanase	*Aspergillus sydowii*	Ca-alginate	Entrapment	([85])
	Bacillus pumilus	Polyurethane foam, scotch brite	Entrapment, Adsorption	([77])
Oxidoreductase and hydrolase	*Pleurotus ostreatus*	Ca-alginate	Entrapment	([86])
α-galactosidase	*Streptomyces griseoloalbus*	Ca-alginate gel	Entrapment	([87])
L- glutaminase	*Pseudomonas*	Ca-alginate	Entrapment	([88])
Polygalacturonase	*Aspergillus niger*	Orange peel	Adsorption	([89])

2.3.2 Fermentation method

The development of the fermentation process is highly significant for the improvement of efficient enzyme production. The fermentation has many methods such as batch, repeated-batch, fed-batch, and continuous fermentation, which are applied for production of the enzyme by the microbial cell.

- Batch fermentation

Batch culture is a closed culture system which contains an initial, and limit amount of nutrient. The inoculated culture is passing through a few phases after inoculation. There is a period during which it appears that no growth has taken place.

- Repeated-batch fermentation

The repeated-batch process is a combination of the fed-batch and batch process, mainly making it possible to conduct the process by long periods and improving the productivity compared to the batch process.

-Fed-batch fermentation

The fed-batch process is characterized by the addition of one or more nutrients to the bioreactor during the process, maintaining the products inside the bioreactor until the final fermentation. The fed-batch processes are amply employed to minimize the effects of the cell metabolism control and prevent any inhibition by substrate or metabolic products.

-Continuous fermentation

Exponential growth in batch culture may be extended by the simultaneous replace of fresh medium into the vessel that, the medium has been designed such that growth is substrate limited and non-toxic limited, exponential growth will proceed until the additional substrate is depleted. This operation may be repeated until the vessel is full. However, if an overflow device were fitted to the fermenter such that the added medium displaced an equal volume of culture from the vessel then continuous production of cells could be achieved. If a medium is fed continuously to such a culture at a suitable rate, a steady state is achieved eventually. Meaning a formation of new biomass by the culture is balanced by the loss of cells from the vessel.

The flow of medium into the vessel is related to the volume of the vessel by the term dilution rate ([90]).

3. Poly (lactic acid) synthesis

3.1 Chemical processes for PLA synthesis

Normally, the commercial PLA is polymerized by chemical process. Lactic acid is a monomer for PLA synthesis. PLA oligomer is intermediary material for PLA-synthesis through two processes: direct polycondensation reaction and ring-opening polymerization. The PLA synthesis of lactic acid is shown in Figure 7 ([61]). Both of two processes necessitate the step of lactic acid pre-polymer production. The lactic acid pre-polymer is PLA with a low molecular weight (Mw=1,000-5,000). The lactic acid pre-polymer is unusable because it has flimsy, crystalline and fragile properties. The direct condensation polymerization, as the polymerization through PLA oligomer formation with the coupling agents. The intention of coupling agents is to increase the molecular weight of the PLA. The formation of the lactic acid pre-polymer by the direct reaction process is mainly used because of the deficiency of reactivity of the end groups, excess water and high consistency of the melted polymer. Ring-opening polymerization, as the lactide formation can be done without the coupling agents. Lactide molecules endure either anionic or cationic ring polymerization, depending on the initiator type ([38]). However, the chemical process requires the extreme condition such as high temperature, high solvent concentrations and high loading of catalysts ([91]). In 2002, Tsuji and Nakahara reported the PLA polymerization was achieved at high temperature (140°C) under acidic condition ([92]). In 2009, Feerzet and Kenji reported the polymerization of PLA by direct polycondensation at 150-250°C for 89 hours under vacuum ([93]). In 2011, Lasprilla and Martinez reported the lactic acid polymerization by ring-opening. It was observed in two steps, in first step was performed at 200°C for 4 hours and followed by 160°C for 24 hours with adding stannous octoate ($Sn(Oct)_2$) as catalyst to promote the esterification reaction under nitrogen condition ([94]).

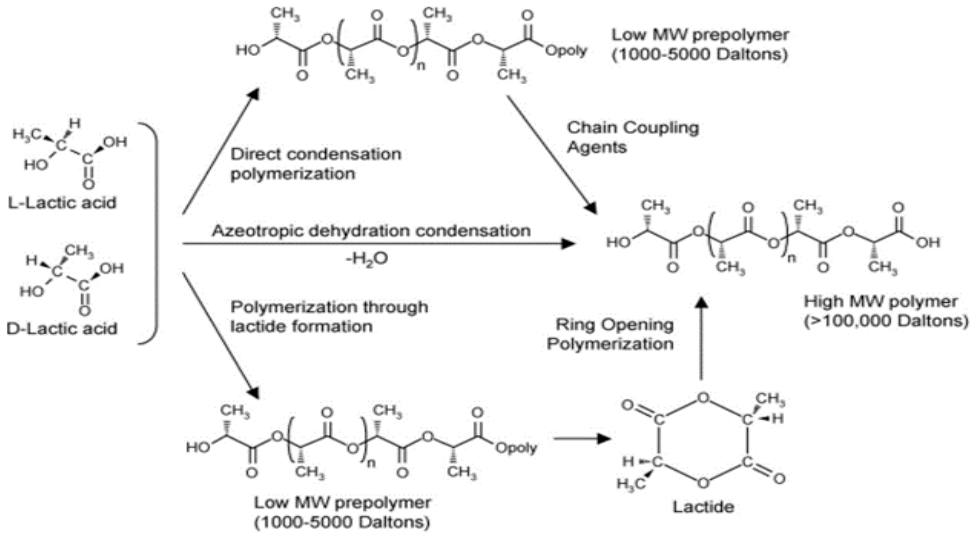


FIGURE 7 PLA synthesized via direct condensation polymerization and ring-opening polymerization to high molecule polymer

The PLA is a semi-crystalline polymer with glass transition temperature around 40 to 80 °C for PLLA, PDLLA and PDLA, melting point of 120 to 178°C, decomposition temperature is 227 to 255°C and swelling in buffer at pH 7.0. Density of amorphous and single crystal PLA are 1.248 and 1.29 g/cm^3, respectively ([95]) as shown in Table 7 ([95]).

TABLE 7 Properties of poly (lactic acid).

Properties	Units	Condition	Value
Degree of crystallinity	%	L-PLA	0-37
Density	g/cm^3	Amorphous	1.248
		Single crystal	1.29
Glass transitions	˚C		
temperature		PLLA	55-80
		PDLLA	43-53
		PDLA	40-50
Melting point	˚C		
		PLLA	173-178
		PDLLA	120-170
		PDLA	120-150
Decomposition temperature	˚C		227-255
Swelling in water	%	pH 7 buffer	2

3.2 The biological process for PLA synthesis

Commonly, PLA is commercially polymerized by chemical processes, which are direct condensation from lactic acid or ring opening polymerization of lactide ([96]). However, the principal disadvantage of the direct condensation are the low molecular weight of derivable PLA because the stability of lactic acid, the lactides and the water produced during the process, which should be consistently removed. The ring-opening polymerization requiring the heavy metals are used as the catalysts such as Zn and Sn oxides, which normally pollute the resultant polymer. Moreover, the high quality of PLA synthesis needs the purity of lactic acid as monomers and under oppressive of temperature and vacuum condition ([20]).

The enzymatic polymerization is an alternative process for PLA synthesis, which has the highest advantages such as better physical and chemical properties of PLA, environment-friendly method, completed under mild conditions, highly specific and

provides enough of the polymerization process. In 2008, Lassalle and Ferreira reported the optimal conditions for biological polymerization at temperature 65°C for 96 hours by lipase under hexane condition ([20]). Chuensangjun et al (2012), reported the molecular weights in the range 2600-4500 Da of PLA was drawn from the commercial lactic acid by the commercial lipase, Lipozyme TL IM at optimum condition temperature 50°C for 5 hours in the toluene solvent and under a nitrogen atmosphere ([21]). Moreover, the PLA synthesized via enzymatic ROP method using free enzyme and immobilized *Candida antarctica* lipase B (CALB) onto chitin and chitosan ([97]). The immobilized lipase B from *Candida antartica* was being used as biocatalyst in ring-opening polymerization of L-lactide at 65°C under supercritical carbon dioxide of a molecular weight (Mw) was 12,900 g/mol ([98]).

4. Biological recycling of PLA

Poly (lactic acid) or PLA is a biodegradable polymer synthesized from lactic acid monomer, which is produced from an agricultural waste via microorganism fermentation process. It has obtained much curiosity because it plays an important role in improving the global warming and the municipal solid waste problem. Therefore, the PLA-degradation and the PLA-polymerization are significant methods. Since 1997, many studies show several isolated and identified PLA-degrading microorganisms. Various PLA- degrading microorganisms have been reported such as bacteria, fungi and actinomycetes as mention in section 3. Moreover, PLA degradation by microbial enzyme is one of an important issue for biological recycling process of PLA.

In addition, PLA is generally polymerized via a chemical process as poly condensation from lactic acid and ring opening polymerization from lactide. Nevertheless, the chemical processes require the extreme conditions, such as high temperature, high concentration of organic solvent and high adding of catalysts. Whereas, the biological polymerization processes showed an alternative process that requires mild conditions and uses an enzymatic method. The enzyme was used for bio-recycling of PLA as lipase. The lipase was used for modification of PLA through bioprocessing that replaced the chemical process. A little report showed the biological

re-polymerization process of PLA using lipase. Jarerat et al. (2006) demonstrated the biological recycling of PLA using enzyme activity by a process that is mild (at relatively low in temperature, 40 °C) and clean (without organic solvent). In addition, a recycling process using the stereospecific enzyme activity of *Amycolatopsis orientalis* at low temperature prevented formation of a mixture of D- and L-lactic acids, which generally occurs in conventional hydrolysis in water at a temperature over 300 °C. The obtained degradation products, monomer and oligomers, can be readily polymerized to high molecular weight PLA by one-step condensation polymerization after the removal of impurities ([56]). Recently, in 2017, Youngpreda et al. investigated the PLA degradation by PLA-degrading enzyme produced from *Actinomadura keratinilytica* strain T16-1 at optimum condition as temperature 60°C for 24 hours released product with 6,843 mg/L of lactic acid subsequently, that degradable products were used as substrate for re-polymerization of PLA by the commercial lipase as catalyst under nitrogen atmosphere for 6 hours. A re-polymerized PLA oligomer was completed with a molecular weight of 378 Da (n=5) ([22]).

The biological process of PLA including the PLA degradation and polymerization were investigated in this study. A section of PLA degradation was performed by using protease produced by *Actinomadura keratinilytica* strain T16-1. Moreover, the thermo-solvent tolerant lipase from *Streptomyces* sp. strain A3301 was applied for re-polymerization of PLA by using a substrate as lactic acid monomer, which was produced from the PLA degradation process in the first section. The re-polymerization process was achieved at the optimized condition under toluene as solvent and nitrogen atmosphere. This thesis was used as a model to complete the biological recycling of PLA as shown in Figure 8 ([23]).

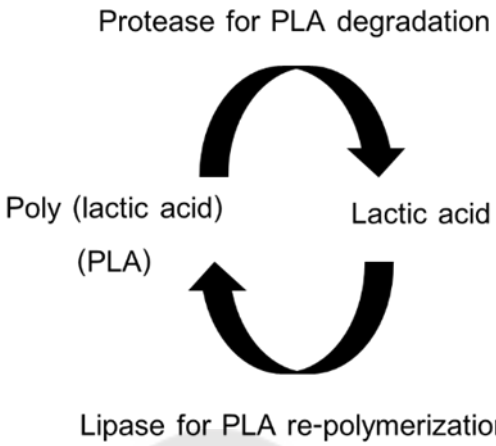


FIGURE 8 The scheme of PLA degradation and synthesis by biological method.

5. Lipase

5.1 Thermo-solvent tolerant lipase

Lipases are the most versatile biocatalysts because they can be used for the synthesis of a wide range of substrates with high stereospecificity and enantioselectivity. Lipases are triacylglycerol ester hydrolases (EC 3.1.1.3), that have a strong ability to catalyze both hydrolytic and synthetic reactions in nature. Lipase is an important biocatalyst because their main functions are hydrolyzing carboxylic ester bonds. Lipase can be esterification, interesterification and transesterification catalyst in non-aqueous media ([99]). Normally, the lipase is generated from microorganisms, animal and plant. Microbial lipase is more interesting due to the occurrence of high yields, highly variable in enzymatic properties and substrate specificity. The applications of lipase are used in food, paper, textile and detergent industries, waste water treatment, fine chemicals, pharmaceuticals and cosmetics production.

Lipases are classified into three classes according to the basic of positional specificity ([100]) as follows:

1. Non-specific lipase

These lipases can hydrolyze the triglyceride, which are converted to free fatty acids and glycerol with the intermediates as mono- and di-glycerides and can displace fatty acid from any the substrate position. The intermediates are more rapidly hydrolysis than triglyceride.

2. 1, 3-specific lipases

These lipases hydrolyze triglycerides at position 1 and 3, which then are converted to fatty acids and cannot hydrolyze ester bond at secondary positions of triglycerides. These lipases can hydrolyze triglycerides to diglycerides faster than those into mono glycerides.

3. Fatty acid-specific lipases

These lipases are fatty acid selectivity and catalyze, that hydrolyze esters which have long-chain fatty acids with double bonds in cis position between C-9 and C-10.

Current developments of biosynthetic applications require non-aqueous environments. The advantages of biocatalysts in organic solvents such as the high dissolution of hydrophobic compounds, displace of thermodynamic equilibrium toward synthesis instead of hydrolysis, minimum water-dependent unwanted reactions, without contamination by microorganisms, modification of enzyme specificity to particular substrates, easy of enzyme recovery and reusability, possible enhancement of thermostability and feasibility of catalyzing reactions that are impracticable in aqueous media ([101,102]). Several organic solvents stability lipases have been reported. Some researchers have discovered this unique property by determining their practical applications, as shown in Table 8.

TABLE 8 Summary of organic solvent tolerant lipase produced by various microorganisms and their stability in organic solvent.

Organism	Stability in organic solvents			Incubation period (h)	Reference
	Stimulatory effect (≥100% relative activity)	Mild inhibitor effect (≥90% relative activity)	Inhibitor effect (<90% relative activity)		
Acinetobacter sp. EH28	15mM, 30mM Dimethylsulfoxide, n-Hexane, acetone	15mM, 30mM Ethanol, 15mM Isopropyl alcohol, DMF	15mM, 30mM Methanol 30mM isopropyl alcohol, DMF	1	([103])
Ralstonia sp. CS274	5-15%(v/v) of Methanol	-	25%(v/v) of glycerol, DMSO, methanol, ethanol, acetone, n-hexane	24	([104])
Streptomyces sp. CS268	25%(v/v) of acetone, benzene, n-hexane, toluene, octane	-	25%(v/v) of ethanol, ethanol, DMSO, acetonitrite	48	([105])

*, **, ***, ****, ***** mean stability in organic solvent at different incubation period

Table 8 (Continued)

Organism	Stability in organic solvents			Incubation period (h)	Reference
	Stimulatory effect (≥100% relative activity)	Mild inhibitor effect (≥90% relative activity)	Inhibitor effect (<90% relative activity)		
Haloarcula sp. G41	35%(v/v) * of glycerol, n-Hexane	-	35%(v/v) ** of DMSO, methanol, ethanol, acetone, *tert*-butanol, chloroform, benzene	<120* <48**	([106])
Idiomarina sp. W33	50%(v/v) *** of n-hexane	50%(v/v) **** of glycerol, cyclohexane	50%(v/v) ***** of DMSO, methanol, ethanol, *tert*-butanol, acetone	>240*** ≥120**** ≤48 *****	([107])
Pseudomonas aeruginosa AAU2	50% (v/v) of iso-octane, xylene	50% (v/v) of cyclohexane	50% (v/v) of n-Heptane, n- Hexane, toluene, benzene, chloroform, dichloromethane, methanol	24	([108])
Pseudomonas aeruginosa LX1	25% (v/v) of n-Hexadecane, iso-octane, glycerol	25% (v/v) of n-Hexane, DMF, DMSO	25% (v/v) of methanol, ethanol, *tert*-butanol, acetone, acetonitrite	48	([109])

5.2 Microbial lipase production

Mostly, lipase is produced by microorganisms as an extracellular enzyme and their production is controlled by the medium composition and physicochemical factors including temperature, pH, agitation speed and dissolved oxygen. A novel organic solvent-stable lipase was produced from *Bacillus subtilis* strain DS9, with the optimum pH and temperature for lipase production at 8.0 and 40°C, respectively of lipase activity 38.8 U/mL ([110]). *Acinetobacter* sp. AU07 produced a thermo- solvent tolerant lipase at temperature 30°C and pH 7.0 with lipase activity of 14.5 U/mL ([111]).The major factors have affected on lipase activity as the medium components such as carbon source because that can be induce to lipase production. The inducers are being used in lipase production such as lipid, triacylglycerols, fatty acids, hydrolyzable esters, Tweens, bile salts and glycerol. The lipidic carbon sources are necessary for obtaining a high lipase yield. In previous reports, A thermophilic bacterium, *Bacillus thermoleovorans* ID-1 grew rapidly in medium containing olive oil, specifically, carbon source with lipase activity of 520U/L ([112]). Lipase production from *Pseudomonas* sp. DMVR46 by using 1% of cotton seed oil as inducer with lipase activity of 48 U/mL ([113]). In addition, nitrogen sources and essential micronutrients is an important factors for microbial growth and production optimization. The requirement of this nutrition for microbial growth is completed by various alternative media as based on its component such as sugars, oils, peptone, yeast extract, malt extract, and agro-industrial residues. A mix of two composition media can be used for the intention of lipase production. Thermo stable lipase was produced from *Pseudomonas* sp. KW1-56 by using medium with 2% of peptone and 0.1% of yeast extract as nitrogen source ([114]). The maximum of lipase production was performed by *Staphylococcus homonis* JX961712 of 19.5 U/mL by using 0.05% of yeast extract as nitrogen source ([113]).

5.3 Purification and characterization of lipase

5.3.1 Purification of lipase

Most of the commercial enzyme is applied in industries need to have high purity, depending on the application such as fine chemicals, pharmaceuticals and cosmetic. The purification system for industrial intentions should be cheap, quick, high-yielding and responsive to scale up operations. They should have the capacity for continuous product recovery, with relatively high potential and selectivity for the desired product. Purification steps is concerned about the culture supernatant concentration consisting of the enzyme by precipitation with ammonium sulfate, ultrafiltration or extraction with organic solvents. Precipitation often gives a higher average yield. The lipase is a hydrophobic in nature, which have large hydrophobic surfaces around the active site. So, the purification of lipase may be completed by affinity chromatography, such as hydrophobic interaction chromatography. The affinity systems can be used at an early stage, but the hydrophobic materials are expensive, so other methods are chosen as the alternative methods such as ion exchange and gel filtration chromatography, there are usually applied after the precipitation step ([115]). The purification of lipase from *Bacillus methylotrophicus* PS3 was observed by using ammonium sulfate precipitation and sephadex G-100 gel column chromatography. The molecular weight of purified enzyme was 31.40 kDa with the purification of lipase was 2.90-fold and final yield 24.21% ([116]). The purification of lipase from *Xanthomonas oryzae* pv. Oryzae YB103 was performed by ammonium sulphate precipitation and phenyl sepharose chromatography with 101.1 of purification fold and 15.7% of yield. The purification of lipase from *Bacillus subtilis* NS8 was achieved by ultrafiltration, DEAE-Toyopearl and sephadex G-75 chromatography with 500 of purification fold and 16% of yield ([115]).

5.3.2 Properties of bacterial lipases

Lipases from many microorganisms have been wildly studied. The properties of lipase are temperature and pH optima, stability, molecular weight, various metal ions and inhibitor tolerant as shown in Table 9 ([100]).

Normally, bacterial lipase has a neutral or alkaline optimum pH and optimum of temperature as the wild range at 30-60°C. Stability in organic solvents is acceptable in synthesis reactions. Generally, lipases are highly stable in several organic solvents such as acetone, ethanol, methanol, and toluene. Effect of metal ions such as cofactors on lipase activity was also reported. Normally, cofactors are not necessary for lipase activity, but divalent cations such as calcium is a stimulator of lipase activity.

TABLE 9 Physiochemical properties of recently studied bacterial lipase

Strain	Mass (kDa)	Optimum pH	Optimum temperature
Burkhoderia ubonensis	33	8.5	65
Streptomyces lividans	31.43	8.0	50
Geobacillus thermodenitrificans	50	9.0	65
Enterobacter sp. Bn12	31.1	8.0	60
Chromohalobacter sp. LY7-8	44	9.0	60
Acinetobacter baylyi	30	8.0	60
Janibacter sp.R02	44	8.0-9.0	80

CHAPTER3
MATERIALS AND METHODS

Equipments

Amicon Ultra-10 centrifugal filters (Merck, USA)

Autoclave (Tomy, Japan)

Biosafety cabinet (NuAire Lab equipment, USA)

Butyl toyopearl 650M resin (Tosoh bioscience, Japan)

DEAE toyopearl 650M resin (Tosoh bioscience, Japan)

Dialysis bag (Spectrapor membrane tubing, Thomas, U.S.A)

Dual range analytical balance (Denver instrument, UK)

Fraction collector (Pharmacia Biotech, USA)

Freeze dryer (Operon, Korea)

Freezer -20ºC (Sanyo, Japan)

Freezer -80ºC (Miracle science, Thailand)

Hot air oven (Thermo Fisher Scientific, USA)

Hot plate (Fisher Scientific, USA)

High temperature shaking incubator (Chemoscience, Thailand)

High temperature incubator (Shellab, USA)

High Performance Liquid Chromatography (Shimadsu, Japan)

InertSustain C18 column (GL Science, Japan)

Laminar flow (Biobase, Germany)

Light microscope (Olympus Optical Co. Ltd, Japan)

Low temperature shaking incubator (LABWIT, Australia)

Magnetic stirrer (Fisher Scientific, USA)

pH meter (Suntex, Thailand)

Polyacrylamide gel electrophoresis eqiupment

Refrigerated centrifuge (TOMY, Japan)

Rotary evaporator (Bushi, Japan)

Scanning electron microscope model (JEOL,USA)

TSK-GEL H_{XL} Series (7.8 mm I.D x 30 cm L) column (Tosoh bioscience, Japan)

3-L stirrer bioreactor (B. Braun Biotech International, Germany)

5-L stirrer bioreactor (B. Braun Biotech International, Germany)

10-L stirrer bioreactor (B. Braun Biotech International, Germany)

UV-Visible spectrophotometer (Shimadsu, Japan)

Vortex (Scientific Industries, Inc., USA)

Water bath (Scientific Equipment source, Canada)

Chemical and media compositions

Acetone (BDH, England)

Acetonitrite (Fisher chemicals, UK)

Acetic acid (VWR, France)

Agar (Himedia, India)

Alginate (Sigma, UK)

Ammonium persulphate (Sigma chemical, UK)

Ammonium phosphate (Thermo Fisher Scientific, USA)

Ammonium sulphate (Emsure®, Germany)

Arabinose (Himedia, India)

Beef extract (Himedia, India)

Bovine serum albumin (Sigma chemical, UK)

Bromophenol blue (Ajax Finechem, Australia)

1-butanol (CARLO ERBA reaganti, France)

Calcium carbonate (SRL, India)

Calcium chloride (Thermo Fisher Scientific, USA)

Casein (Himedia, India)

Casein enzyme hydrolysate (Himedia, India)

Cellulose (Himedia, India)

Commassie Brilliant Blue (PanReac AppliChem, Germany)

Copper sulfate pentahydrate (Sigma chemical, UK)

Chloroform (RCI Labscan Limited, Thailand)

Dextrose (Thermo Fisher Scientific, USA)

Dichloromethane (RCI Labscan Limited, Thailand)

Dimethyl sulfoxide (Fluka chemika, Switzerland)

Dipotassium phosphate (QRecTM, New Zealand)

Disodium phosphate (QRecTM, New Zealand)

Ethanol (Alcoh-A, Thailand)

Ethyl acetate (Fisher chemicals, UK)

Ethylenediaminetetraacetic acid (Ajax Finechem, Australia)

Galactose (Bacto®, Australia)

Gelatin (Himedia, India)

Glycerol (Thermo Fisher Scientific, USA)

Gum Arabic (Sigma chemical, UK)

Hexane (J.T. baker® chemicals, USA)

37% Hydrochloric acid (Emsure®, Germany)

Iron(II) Sulfate heptahydrate (Merck, USA)

Isoamyl alcohol (J.T. baker® chemicals, USA)

Isopropanol (J.T. baker® chemicals, USA)

L-asparagine (Sigma chemical, UK)

Lactic acid (CARLO ERBA reaganti, France)

Lactose (Merck, USA)

Lysozyme (Sigma chemical, UK)

Manganese(II) chloride tetrahydrate (Fluka chemika, Switzerland)

Magnesium sulphate Heptahydrate (Thermo Fisher Scientific, USA)

Malt extract (Himedia, India)

Maltose (APS, Australia)

Mannitol (Thermo Fisher Scientific, USA)

Mannose (Fluka chemika, Switzerland)

Methanol (RCI Labscan Limited, Thailand)

Mercaptoethanol (Sigma chemical, UK)

Mono ammonium phosphate (Sigma chemical, UK)

Peptone (SRL Pvt. Ltd., India)

Phosphoric acid (RCI Labscan Limited, Thailand)

Phenol (Rankem, India)

2- Nitrophenol (Sigma chemical, UK)

p-Nitrophenyl palmitate (Sigma chemical, UK)

Polyacrylamine (PanReac Applichem, Germany)

Potassium dihydrogen phosphate (QRecTM, New Zealand)

Potassium nitrate (BDH, England)

Raffinose (Sigma chemical, UK)

Sodium acetate (SRL, India)

Sodium carbonate (Thermo Fisher Scientific, USA)

Sodium chloride (Thermo Fisher Scientific, USA)

Sodium dodecyl sulfate (ALDRICH, USA)

Sodium hydrogen phosphate (SRL, India)

Sodium hydroxide (QRecTM, New Zealand)

Sodium lactate (Sigma chemical, UK)

Sodium nitrate (CARLO ERBA reaganti, France)

Soluble starch (SRL, India)

Sorbitol (Bio Basic Inc., Canada)

Soy peptone (Himedia, India)

Tetrahydrofuran (J.T. baker® chemicals, USA)

Tetramethylethylenediamine (Sigma chemical, UK)

Toluene (J.T. baker® chemicals, USA)

Tris hydroxymethyl-methylamine (Thermo Fisher Scientific, USA)

Triton X 100 (EMD Chemicals Inc., Germany)

Tryptone (Himedia, India)

Tween 80 (QRecTM, New Zealand)

Xylose (SRL, India)

Yeast extract (Himedia, India)

Zinc sulfate heptahydrate (Ajax Finechem, Australia)

Methods

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

1.1 Microorganism and inoculum preparation

The actinomycete, *Actinomadura keratinilytica* strain T16-1 was isolated and identified by Sukkhum et al., 2009 ([10]) and kept at NITE Biological Resource Center (NBRC), japan and Biotec Culture Collection (BCC), Thailand.

Inoculum preparation was performed by streaking the strain onto ISP2 agar (International Streptomyces project-2 medium) (Appendix A1) and incubating at 45°C, for 3 days. The strain was sub-cultured in 250 mL Erlenmeyer flask containing 50 mL ISP-2 broth and incubated at 45°C with rotary shaking at 150 rpm for 2 days. The cells were harvested by filtration through Whatman No.1 filter paper, washed twice and re-suspended in 85% NaCl and adjusted at OD600 as 0.9, and used as inoculum.

1.2 Substrate preparation and fermentation medium

Poly (DL-lactic acid) or 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 for PLA degrading enzyme production. The PDLLA powder was prepared by adding 30 mL of dichloromethane into a 0.3 g PDLLA pellet and mixing until PDLLA pellet completely dissolved. Precipitation of the PDLLA powder by adding 200 mL of methanol to the PDLLA solution, which was then air-dried and used as the substrate. Then, the 10% (v/v) of inoculum was inoculated into the fermentation medium (Appendix 2). The culture flasks were incubated at 45°C, 150 rpm for 3 days. The enzyme was collected by filtration through Whatman No.1 filter paper, and the filtrate was further analyzed for enzyme activity.

1.3 Bacterial cell immobilization

The seed culture for cell immobilization was prepared as describe above. Various immobilizer such as luffa disc, sponge (Scotch BriteTM scrubbing sponge, 3M Thailand, Bangkok Thailand) and scrub pad (Scotch BriteTM 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% (v/v) 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 re-suspended in 0.85% NaCl and then added into 2% sodium alginate at a final concentration of 10%. The cell suspension was dropped into 0.1 M $CaCl_2$ solution under stirring slowly. The calcium alginate beads were collected, washed twice with 0.85% NaCl solution and transferred to sterile basal medium. All flasks were incubated at 45°C, 150 rpm for 7 days. The samples were taken at 24-hours intervals and collected by filtration through Whatman No. 1 filter paper. The filtrate was further analyzed for enzyme activity.

1.4 Batch fermentation in 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 as shown in Figure 9. The dissolved oxygen (DO) and pH probes were positioned at the top of the fermenter. The 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 minutes. 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 vvm) according to the experimental set up. The culture had been sampling at 6-hours interval for the PDLLA-degrading activity assay. The culture broth was obtained by filtration through Whatman No.1 filter paper.

1.5 Repeated batch fermentation

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 5-L stirrer fermenter with 4-L working volume. Then, the mixture was inoculated with 10% (v/v) of cell suspension and the fermentation proceeded at 170 rpm, 0.25 vvm and 45°C for 48 hours. After that, the culture broth was completely removed from the fermenter and re-suspended 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-hours sampling interval. The culture broth was obtained by filtration through Whatman No.1 filter paper.

1.6 Continuous process

The continuous process for PDLLA-degrading enzyme production was performed by culturing strain T16-1 in 5-L stirrer fermenter with 4-L of basal medium containing 0.035% (w/v) PDLLA and 0.1% (w/v) scrub pad as immobilizer. A 10% (v/v) cell suspension was used and 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/hours. The culture was sampled at 6-hours intervals for PDLLA degrading enzyme activity assay. The culture broth was obtained by filtration through Whatman No.1 filter paper.

1.7 Batch fermentation in 10-L stirrer fermenter

The PDLLA-degrading enzyme production was scaled up to a 10-L stirrer fermenter with 7-L working volume (B. Braun Biotech Biostat B, Sartorious, Goettingen, Germany). A 7-L volume of basal medium containing 0.035% (w/v) PDLLA and 0.1% (w/v) scrub pad were added into the fermenter and sterilized at 121°C for 30 minutes. A 10% (v/v) cell suspension was inoculated and fermentation was performed at an agitation speeds of 170 rpm, aeration rate of 0.25 vvm and 45°C for 48 hours. The culture was sampled at 6-hours intervals for PDLLA degrading enzyme activity assay. The culture broth was obtained by filtration through Whatman No.1 filter paper.

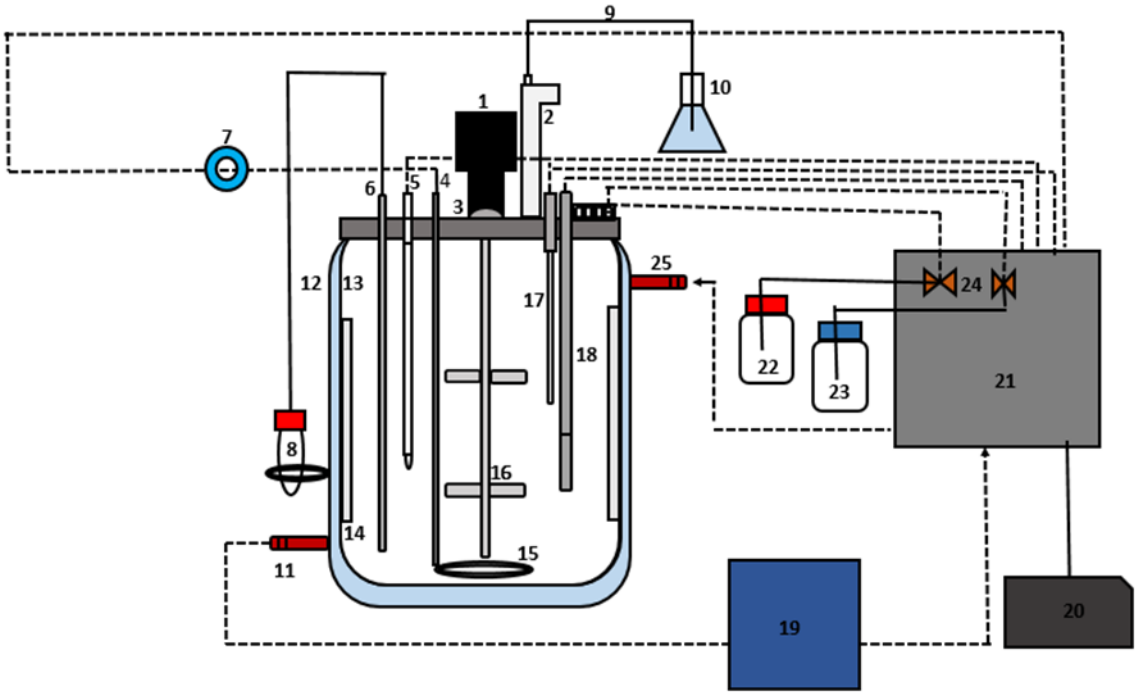


FIGURE 9 Schematic diagrams of 5-L stirrer bioreactor used throughout this study. Experiment apparatus: 1. Motor; 2. Condenser; 3. Inoculum port; 4. Air inlet line; 5. pH probe; 6. Sampling line; 7. Air filter; 8. Sampling port; 9. Air outlet line; 10. 70% ethanol; 11. Water outlet line; 12. Water jacket; 13. Vessel; 14. Baffles; 15. Air sparger; 16. Impeller; 17. Antiform probe; 18. DO probe; 19. Cooler; 20. Air pump; 21. Controller; 22. 2M HCl; 23. 6M NaOH; 24. Pump and 25. Water inlet line

1.8 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) (Appendix B1) by 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 minutes. The reaction was stopped by cooling on ice for 5 minutes. 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.

1.9 Scanning electron microscopy analysis

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

1.10 Calculation

The PDLLA-degrading enzyme production efficiency was calculated in terms of enzyme yield ($Y_{p/s}$) and enzyme productivity (Q_p).

$$\boldsymbol{Yield(Y_{P/S}) = \frac{Enzyme\ activity\ (U/ml)}{substrate\ concentration\ (g/ml)}} \quad \text{——} \quad (1)$$

$$\boldsymbol{Enzyme\ productivity\ (Q_p) = \frac{Enzyme\ activity\ (\frac{U}{ml})}{Fermentation\ time\ (h)}} \quad \text{——} \quad (2)$$

1.11 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.

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

2.1 Poly (DL-lactic acid) degrading enzyme production

PDLLA-degrading enzyme was produced by *A. keratinilytica* strain T16-1 under the condition as describe in section 1. The crude enzyme was subjected to use in the study of PDLLA degradation process.

2.2 Optimization of PDLLA degradation in a 5-L bioreactor

The crude enzyme with an initial activity of 1,000 U/mL was diluted to 200 U/mL by using 100 mM Tris-HCl at a pH 9.0. The reaction mixture, including 4 g/L PDLLA powder and 3.5-L of diluted enzyme, was added into a 5-L stirrer 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 hours for 5 days. The sample was collected by centrifugation at 10,000 × g for 5 minutes at 4°C and subjected to analyze the lactic acid concentration by High Performance Liquid chromatography (HPLC, Shimadsu, Japan). The factors affecting PLA degradation was determined in a 5-L bioreactor. The effect of various agitation speeds on PDLLA 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 PDLLA degradation was performed 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 an HPLC method and used for a calculation of percentage conversion efficiency. All experiments were performed in triplicate.

2.3 The inhibition effect of lactic acid on PDLLA degradation

The effect of lactic acid concentration on PDLLA degradation was determined by adding various lactic acid concentrations into the PDLLA degradation mixture. The reaction mixture contained 1 g of PDLLA powder, 25 mL of 200 U/mL crude enzyme and various final lactic acid concentrations (2,000, 4,000, 6,000 and 8,000 mg/L) and added into 100ml Erlenmeyer flasks. The suspension was incubated at 60°C and shaking at 50 rpm for 48 hours using incubator shaker. The samples were taken every 6 hours, and the final lactic acid concentration was determined by using HPLC.

The controlled experiment was conducted by using the reaction mixture described above without adding lactic acid. All experiments were done in triplicate.

2.4 The effect of the lactic acid removal method on PDLLA degradation

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

A. Control experiment

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

B. Separating PDLLA-degradation and dialysis

One gram of PDLLA powder was added into 25 mL of 200 U/mL crude enzyme in 100 ml Erlenmeyer flasks. The reaction mixtures were incubated at 60°C and shaken at 50 rpm for 24 hours using incubator shaker. All of the suspension was moved into a dialysis bag (Mw= 10 kDa cutoff) and soaked in 250 ml beaker containing 200 ml of 100 mM Tris-HCl buffer at pH 9.0 and 4°C for 24 hours. The experiments were done in triplicate. The dialyzed enzyme was subjected to repeated degradation of PDLLA powder by using the same condition for 24 hours. The samples were achieved after degradation and the lactic acid concentration and PDLLA weight loss were determined.

C. Simultaneous PDLLA-degradation and dialysis

One gram of PDLLA powder and 25 mL of 200 U/mL crude enzyme were added to the dialysis bag. The dialysis bag was soaked in 250 ml beaker containing 200 ml of 100 mM Tris-HCl buffer at pH 9.0 and incubated at 60°C and shaken at 50 rpm for 48 hours using incubator shaker. The buffer was changed every 24 hours. The experiments were done in triplicate. The degradation products were obtained after

centrifugation at 10,000 × g and 4°C for 5 minutes. The lactic acid concentration was determined by using HPLC. The percentage of PDLLA weight loss was analyzed.

2.6 Commercial PLA product degradation by crude PDLLA-degrading enzyme

One gram of PLA product, PLA tray (90% polylactic acid, size of 0.3×1 cm and thickness of 0.1 mm), spoon, tube, glass, cup lid (100% polylactic acid, size of 0.3×1 cm and thickness of 0.1 mm) and PLA textile and 25 mL of 200 U/mL crude enzyme were added to a dialysis bag, soaked in 250 ml beaker containing 200 ml of 100 mM Tris-HCl buffer at pH 9.0 and incubated for 48 hours at 60°C and shaken at a speed of 50 rpm using incubator shaker. The buffer was changed every 24 hours. The degradation products were obtained after centrifugation at 10,000 × g, 4°C for 5 minutes. All experiments were performed in triplicate. The lactic acid concentration was determined by using HPLC. The percentage of PLA weight loss was analyzed.

2.7 Analytical method

The PDLLA-degrading enzyme activity was measured by the method in previous section 1.8. The lactic acid concentration in the reaction mixture was analyzed by HPLC (InertSustain C18 column with a mobile phase of 10 mM $NH_4H_2PO_4$ (pH 2.6 with H_3PO_4), a flow rate of 1.0 mL/minutes, and a temperature of 40°C). The percentage of PDLLA converted to liberate lactic acid (% conversion efficiency) was calculated according to the method of Youngpreda et al ([22]) and the weight loss was determined through the process drying the PDLLA residual at 100°C overnight.

The percentage of conversion efficiency was calculated using the equation (3-4).

$$Liberated\ lactic\ acid\ (g) = \frac{initial\ weight\ of\ PDLLA\ powder\ (mg) \times Mw.\ of\ lactic\ acid\ (mL) \times 597}{Mw\ of\ PDLLA} \quad (3)$$

$$Conversion\ efficiency\ (\%) = \frac{Liberated\ lactic\ acid\ \left(\frac{g}{L}\right) \times total\ volume\ (mL) \times 100}{1{,}000\ \times theoretical\ lactic\ acid\ (mg)} \quad (4)$$

3. Thermo-solvent tolerant lipase for PDLLA polymerization.

3.1 Isolation of thermo-solvent tolerant lipase producing actinomycetes for PDLLA repolymerization

Seventy-seven soil samples were collected from Sakerat Environmental Research Station Sakerat Biophere Reserves (SERS), Pakthongchai District, Nakhon Ratchasima Province, Thailand and preserved at 4°C until the experiment use. One-gram of each soil was added into 50 mL enrichment medium (Appendix A3) and incubated at room temperature (30°C), 150 rpm for 2 days. The pre-enrichment medium was diluted with 0.85% (w/v) NaCl, spread on tween 80 agar plate (Appendix A4) and incubated at room temperature for 7 days. Lipase producing actinomycetes that appeared in the precipitation zone on tween 80 agar were selected and streaked twice on ISP2 agar (Appendix A1). The other method for screening lipase producing actinomycetes was phenol pretreatment method. The soil samples were dried at 120°C for 1 hours, suspended in 1.5% (w/v) phenol solution, incubated at 30°C for 30 minutes, diluted with 0.85% (w/v) NaCl. Subsequently, cultured on starch casein agar (Appendix A5) and incubated at room temperature for 7 days. The isolated actinomycetes were streaked on ISP2 agar. The pure cultures from both of with and without pretreatment methods were tested for lipase activity by spotting on tween 80 agar plate.

The isolates that presented the extension of precipitation zone was selected as potential producing strain and used for further studies. The ability of lipase production was calculated by the enzyme activity index using the equation (5).

$$Lipase\ activity\ index = \frac{Diameter\ of\ precipitation\ zone\ (cm)}{Diameter\ of\ colony\ (cm)} \quad \text{——(5)}$$

3.2 Screening of solvent tolerant lipase producing actinomycetes

Lipase producing actinomycetes were cultured on ISP2 agar, incubated at room temperature for 7 days. The isolate were cultured in ISP-2 broth and incubated at room temperature, 150 rpm for 2 days. The cells were harvested by centrifugation at 10,000 g for 10 minutes and washed twice with 0.85% (w/v) NaCl. The initial cell density of inoculum was adjusted the turbidity at OD 600 of 0.9. Afterwards, 10% (v/v) inoculum

was inoculated into 45 mL production medium (Appendix A6) containing 10% (v/v) organic solvent (toluene or hexane: chloroform (ratio 2:1)) and incubated at room temperature, 150 rpm for 3 days. The culture broth was obtained by centrifugation at 10,000 g, 4°C for 30 minutes. The supernatant was used as the crude enzyme activity assay. The growth of the isolates were determined by dropping on ISP-2 agar and incubated at room temperature for 3 days. The result was recorded as follow:

Strongly positive growth (+++), when the isolated actinomycete growth on ISP-2 agar is greater than a control.

Positive growth (++), when the isolated actinomycete growth on ISP-2 agar is equal to a control.

Doubtfully growth (+), when the isolated actinomycete growth on ISP-2 agar is less than a control.

Negative growth (-), when there isn't the isolated actinomycete growth on ISP-2 agar.

The isolated actinomycete was cultured in production medium without solvent as a control experiment.

The isolates that can grow on ISP-2 agar plate with organic solvent and showed high enzyme activity was selected for the further studies.

3.3 Screening of thermostable solvent tolerant lipase

3.3.1 Crude lipase preparation

Lipase producing actinomycetes were cultured on ISP2 agar, incubated at room temperature for 7 days. The isolates were cultured in ISP-2 broth and incubated at 30°C, 150 rpm for 2 days. The cells were harvested by centrifugation at 10,000 for 10 minutes and wash twice with 0.85% (w/v) NaCl. The initial cell density of inoculum was adjusted the turbidity at OD 600 of 0.9. Afterwards, 10% (v/v) inoculum was inoculated into 45 mL production medium (Appendix A6) incubated at 30°C, 150 rpm for 3 days. The culture broth was collected by centrifugation at 10,000 g, 4°C for 30 minutes. The supernatant was used as the crude enzyme for the thermo-solvent tolerant test.

3.3.2 Effect of temperature on crude lipase activity and stability

The thermostability of the crude enzyme was tested by incubating at different temperatures (37, 45, 55 and 65°C) for 1 hour before testing the remaining enzyme activity. The residual lipase activity was measured by conducting the reaction at temperature 37°C, pH 8.0 for 10 minutes. The activity of the enzyme was considered as 100% under standard assay conditions.

3.3.3 Effect of organic solvent on lipase stability

The crude enzyme solutions were incubated with 10-30%(v/v) of different organic solvents (Toluene or Hexane: chloroform ratio (2:1)) at 30°C, 200 rpm for 1 hours. The remaining lipase activity was estimated against the control, without solvent added.

3.3.4 Assay of lipase activity

The lipase activity was determined by a spectrophotometric assay using *p*-nitrophenylpalmitate (*p*NPP) as a substrate. The reaction mixture consisted of 0.2 mL of enzyme solution, 0.2 mL of 0.3 mg/mL substrate (3 mg *p*NPP dissolved in 1 mL isopropanol and 9 mL of 50 mM phosphate buffer pH 8.0). The reaction mixture was kept at 37°C for 10 minutes, boiled in water for 5 minutes, added 1.6 mL distilled water to stop the reaction and determined at optical density at 410 nm. *p*-nitrophenol was used as standard at a concentration range between 0-10 µmol per mL. One unit of lipase activity was defined as the amount of enzyme which liberated 1 µmol of *p*-nitrophenol per minutes under assay condition.

3.4 Identification of thermo-solvent tolerant lipase producing actinomycetes.

3.4.1 Morphological characterization

The isolate A3301 that had the highest ability to produce the thermo-solvents tolerant lipase was selected for further. The strain A3301 was cultured on ISP-2 agar and incubated at room temperature for 7 days for gram's stain determination. The morphology of the isolate was measured under microscope at objective lens of 40X and 100X after incubated at room temperature for 7 days.

Cultural characterization of lipase producing actinomycetes on various kinds of media were determined according to the method of Shirling and Gottlieb (1996). To examine the degree of growth, pigmentation and color of colony, the strain was grown on yeast-malt extract agar (International Streptomyces Project (ISP-2)) , oatmeal agar (ISP-3) (Appendix A7), inorganic salt-starch agar (ISP-4) (Appendix A8) and glycerol-asparagine agar (ISP-5) (Appendix A9). The plate was observed plate at 7, 14 and 24 days and incubated at room temperature. Color of colony and pigmentation determination was recorded only for mature cultures.

3.4.2 Physiological and biochemical characterization

The strain A3301 was subjected to study physiological and biochemical characterization such as utilization of the various carbon sources, NaCl tolerance, ability to degrade substrates and enzyme production.

Utilization of carbon sources was tested by using modified Pridham and Gottlieb medium. Carbon sources for this test were without carbon source (negative control), dextose (positive control), xylose, mannose, galactose, maltose and lactose. They were sterilized without heat by one of the following methods: Filters sterilize 10% solution through bacteriological filter. Sterilized carbon sources will be added into the ISP-9 agar (Appendix A10) at a final concentration of 1% (w/v) at 60°C. The mixture was agitated 25 mL of medium per dish was poured into Petri dishes. The duplicated plate of each carbon compound was prepared for each culture to be tested. Approximately 0.05 mL of washed inoculum will be placed onto one edge of the agar surface and streaked straight across the dish. Repeat with a second drop. Inoculate duplicate plates. All tested plates were incubated at room temperature and observed the results after 3 days intervals for 14 days.

The growth from a given carbon source was compared with the two controls; growth on ISP-9 alone, and growth on ISP-9 plus glucose. The results will be recorded as follows:

- Strongly positive utilization (+++), when growth on tested carbon in ISP-9 medium is equal to or greater than growth on ISP-9 medium plus glucose

- Positive utilization (++), when growth on tested carbon is significantly better than on ISP-9 without carbon, but somewhat less than on glucose.

- Utilization doubtful (+), when growth on tested carbon is only slightly better than on the ISP-9 without carbon and significantly less than with glucose

- Utilization negative (-), when growth is similar to or less than growth on ISP-9 medium without carbon.

NaCl tolerance and growth temperature was assessed using ISP-2 medium. Washed inoculums was prepared. An approximately 0.05 mL of washed inoculums was placed onto one edge of the agar surface. Then, it was streaked straight across the dish and repeated with a second drop. Inoculate duplicate plates. Plates was observed the results after 3 days intervals for 14 days.

The growth was compared with a control; growth on 0% NaCl in ISP-2 medium. The result was recorded as follow:

- Positive growth (++) , when growth on tested NaCl concentration in basal medium is equal to or greater than growth on 0% NaCl in ISP-2 medium.

- Growth doubtful (+) , when growth on tested NaCl concentration in ISP-2 medium is somewhat significantly less than on 0% NaCl in ISP-2 medium.

- Negative growth (-) , when there is no growth on tested NaCl concentration in ISP-2 medium.

The strain was examined for the ability to degrade substrates. Various substrates will be used to determine the ability to degrade lipid, cellulose, gelatin, skim milk, nitrate from nitrite. Place approximately 0.5 mL of washed inoculum into each plate and incubated at room temperature for 7, 14 days. For observation cellulose degradation, the clear zone on CMC agar (Appendix A11) when used Congo-red as reagent test. In case of gelatin (Appendix A12) liquefaction, after incubate the strain; the medium was kept in refrigerator for 30 minutes. The positive result, gelatin become liquefies after kept in refrigerator compared with control. Observation of casein degradation, the positive result is the clear zone on skim milk agar plate (Appendix A13) . Reduction of nitrate to nitrite was observed by

using GR reagent (the positive is color change to red or pink) and Zn powder. Starch, the hydrolysis test was observed clear zones on the ISP4 plate.

3.4.3 Phylogenetic relationship based on 16s rDNA sequencing

The strain A3301 studied phylogenetic relationship based on 16s rDNA sequencing. Genomic DNA was extracted from the strain as described by Hopwood et al., (1985) (Appendix B2). One gram (wet weight) of mycelium was suspended in 5 mL of Tris-EDTA (TE) buffer, added 10 mg/ml of lysozyme, gently mixed until completely dissolved and incubated at 37°C for 3 hours. After that, the reaction mixture was mixed with 80 µl of 10% SDS, incubated at 55°C for 30 minutes. The genomic DNA was purified by adding 400 µl of phenol: chloroform: isoamyl alcohol, mixed gently for 5 minutes and centrifuged at 13,000 rpm, 4°C for 15 minutes. The upper phase was carefully transferred with a shortened Pasteur pipette into a fresh screw cap bottle. The step of phenol: chloroform: isoamyl alcohol extraction was repeated twice and the upper phase was carefully transferred into a new screw cap bottle. Three molar of sodium acetate was added at ratio 1:10 of the supernatant and followed by adding 1 mL of absolute ethanol and centrifuged at 13,000 rpm, 4°C for 15 minutes. The supernatant was removed and DNA was air dried overnight. The DNA was suspended with 500 µl of TE buffer and stored at 4°C. After that, 16s rDNA amplification was determined by using 5 µl of genomic DNA as DNA template for PCR. Subsequently, the reaction mixture consisted of the universal primers as 27F (5'-AGAGTTTGATCCTGGCTCAG –3') and 1492 (5'- GGTTACCTTGTTACGACTT-3'). The PCR conditions with 25 PCR cycle were done at 94°C for 15 seconds, 50°C for 30 seconds, and 72°C for 1 minutes 30 seconds. This was followed by one cycle of 2 minutes at 72°C and hold at 4°C. After that, the PCR products was examined by electrophoresis, purified and sequenced. A homology search was performed using Genbank database. For further characterization of the strain A3301, a neighbor-joining phylogenetic tree was constructed with the MEGA 7.0 program.

3.5 Optimization of lipase production by strain A3301

3.5.1 Inoculum preparation

The strain A3301 was cultured on ISP-2 agar, incubated at room temperature for 7 days. The strain was cultured in ISP-2 broth and incubated at 30°C, 150 rpm for 2 days. The cells were harvested by centrifugation at 10,000 for 10 minutes and washed twice with 0.85% (w/v) NaCl. The initial cell density of inoculum was adjusted the turbidity at OD 600 of 0.9.

3.5.2 Selection of production medium formulations

The lipase production by the strain A3301 was investigated in various medium formulations as listed below:

Medium A, the modifications medium was designed by Cho S.S. (2012)([117]): (w/v) glucose, 10g; soybean, 10g; Na_2HPO_4 0.1 g and adjusted to 1-L with DW.

Medium B, the medium was YR medium used rice bran oil as carbon source and inducer: (w/v) yeast extract, 10g; NaCl, 5g; rice bran oil, 3 % (v/v) and adjusted to 1-L with DW.

Medium C and medium D is Medium A with oil cake as inducer at 10 and 30 g/L (w/v).

3.5.3 Effect of various factors on lipase production.

3.5.3.1 Effect of incubation period on lipase production

Ten % (v/v) inoculum was added into production medium A and incubated at 30°C, 150 rpm. The culture broth was harvested at 24-hours intervals for 7 days by centrifugation at 10,000 rpm, 4°C for 30 minutes. The collected supernatant was used as crude enzyme solution and subjected to assay for enzyme activity.

3.5.3.2 Effect of different carbon sources on lipase production

At the initial production medium, glucose was presented as a carbon source for lipase production. After that, it was replaced by different carbon sources such as xylose, mannose, galactose, maltose and lactose at a final concentration of 1% (w/v) while keeping the other parameters constant.

3.5.3.3 Effect of carbon concentrations on lipase production

To study the optimum carbon source concentration for lipase production, different concentration of carbon source (0-3% (w/v)) were added into production medium while keeping the other parameters constant.

3.5.3.4 Effect of different nitrogen sources on Lipase production

Soybean in the production medium was replaced by different nitrogen sources such as malt extract, tryptone, peptone, yeast extract, $NaNO_3$, $(NH_4)_2SO_4$ and KNO_3 at a final concentration of 1% (w/v) while keeping the other parameters constant.

3.5.3.5 Effect of nitrogen concentration on Lipase production

To study the optimum nitrogen source concentration for lipase production, different concentration of nitrogen source (0-3% (w/v)) were added into production medium while keeping the other parameters constant.

3.5.3.6 Effect of temperature on Lipase production

The optimum temperature for lipase production was investigated at different temperatures between 30-55°C while keeping the other parameters constant.

3.5.3.7 Effect of initial pH on Lipase production

The optimum pH for lipase production was demonstrated at various pH between 5.0-10.0 while keeping the other parameters constant.

3.6 Purification and characterization of lipase

3.6.1 Purification of lipase

The enzyme solution was adjusted to 20% saturation of $(NH_4)_2SO_4$ by adding $(NH_4)_2SO_4$ with a concentration of 106 g/L (w/v). The enzyme solution was directly applied to Butyl Toyopearl 650M column. The column was equilibrated with 100 mM Tris-HCl pH 9.0 containing 20% saturation of $(NH_4)_2SO_4$ at flow rate of 2 mL/minutes and decreasing gradient elution with 100mM Tris-HCl containing 20-0% saturation of $(NH_4)_2SO_4$. All fractions determined protein content (Appendix B3) and lipase activity. The active fractions were pooled and applied into DEAE 650M anion-exchange chromatography. The column was equilibrated with 100 mM Tris-HCl pH 9.0 at flow rate of 2 mL/minutes and increasing gradient elution with 100mM Tris-HCl

containing 0-2 M NaCl. All fractions determined protein content and lipase activity. The fraction with the highest lipase activity were concentrated with 10kDa Amicon ultra-4 Centrifugal Filters by ultracentrifugation at 3,000 g, 4°C for 3 hours. The supernatant from each step was subjected to analyze of molecular mass of protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

3.6.2 Determination of molecular mass of protein

The molecular mass of purified enzyme was examined by SDS-PAGE as described by Laemmli using a 4% (w/v) stacking and 14% (w/v) polyacrylamide separating gels. Subsequently, the gel was stained with Coomassie Brilliant Blue (CBB) R-250 and destained. (Appendix B4) A low range protein marker kit was being used as the mass calibration with the molecular weight range of 17-250 kDa.

3.6.3 Characterization of purified lipase

3.6.3.1 Effect of temperature on lipase activity and stability

The effect of temperature on lipase activity was determined by incubating reaction mixture with purified enzyme at different temperature in the range of 30-70°C at 0.1M phosphate buffer pH 8.0. The activity was measured by the method describe above. The maximum lipase activity was set at 100% relative activity. The thermostability of purified enzyme was examined by pre-incubating the purified enzyme at different temperature in the range of 30-70°C for 30 minutes. Subsequently, the pre-incubated enzyme was determined the lipase activity at optimum condition.

3.6.3.2 Effect of pH on lipase activity and stability

The effect of pH on lipase activity was performed by using different pH range as 5.0-10.0 (0.1M acetate buffer pH 3-5, 0.1M phosphate buffer pH6-8 and 0.1M glycine-NaOH buffer pH 9-10). To determine pH stability, the purified enzyme was pre-incubated at various pH (5.0-10.0) at 45°C for 1 hours. Afterwards, the pre-incubated enzyme was being measured for the residual lipase activity at optimum condition. The maximum lipase activity was set as 100% relative activity.

3.6.3.3 Effect of metal ions, surfactants and enzyme inhibitors on lipase stability

The effect of metal ions (Li^{+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, Co^{2+}, Fe^{2+}, Cu^{2+}, Ni^{2+} and Ba^{2+}), surfactants (SDS, Tween 85 and Triton X-100) and enzyme inhibitors (EDTA) on lipase stability were determined the purified enzyme was pre-incubated with 1mM metal ions, 1mM enzyme inhibitor and 0.05% surfactant at 45°C for 1 hours. Afterwards, the pre-incubated enzyme determined the enzyme activity at optimum condition. The controlled reaction (without adding metal ions, surfactants and inhibitor) was being used as 100% relative activity.

3.6.3.4 Effect of organic solvent on lipase stability

The effect of various organic solvents such as acetone, chloroform, toluene, 1-butanol, isopropanol, methanol, ethanol, acetonitrite, ethyl acetate and DMSO on lipase stability was assayed by incubating purified enzyme with 25% of organic solvent at 45°C for 1 hours. Then, the incubated enzyme was being examined enzyme for activity through standard method as describe above. The controlled reaction (without adding organic solvent) was used as 100% relative activity.

4. Re-polymerization of PDLLA by crude thermo-solvent tolerant lipase produced by strain A3301

4.1 The PDLLA polymerization process

The PDLLA polymerization was performed under condensation system. The commercial lactic acid was mixed with toluene at a final concentration of 450 mg/ml, heated to 60°C, and then 10% (w/v of lactic acid) of the dried enzyme produced by strain A3301 was added into the solution at 10% (w/v of lactic acid). The two phases reaction mixture was continuously stirred at 60°C under a nitrogen atmosphere for 6 hours. After that, toluene was removed by evaporation at 60°C. The PDLLA product was extracted by adding 50 mL of dichloromethane, shook at 150 rpm for 1 hours, the dichloromethane phase was transferred to a fresh flask and dichloromethane was removed by evaporation at 60°C. The precipitate at the bottom of evaporation flask was

dissolved with tetrahydrofuran (THF). The polymerized PDLLA product was being used to determine the molecular weight of the polymer.

4.2 Preparation of thermo-solvent tolerant enzyme

The thermo-solvent tolerant lipase for PDLLA re-polymerization was dried by using freeze dried process. The supernatant of thermo-solvent tolerant lipase produced by *Streptomyces* sp. strain A3301 was froze by keeping in freezer -80°C (Miracle science, Thailand) for 24 hours. Subsequently, froze enzyme was dried in freeze drier for 2 days. The dried lipase was used as biocatalyst in the reaction mixture of PDLLA re-polymerization.

4.3 Optimization of PDLLA polymerization by crude lipase using lactic acid as monomer

4.3.1 Effect of reaction time on PDLLA polymerization

The reaction mixture was demonstrated at different reaction time (8, 16 and 24 hours.) at 60°C using polymerization process as shown above.

4.3.2 Effect of temperature on PDLLA polymerization

The reaction mixture was investigated at various temperatures (50, 60 and 70°C) for 8 hours using polymerization process as shown above.

4.3.3 Effect of concentration of enzyme on polymerization

The freeze dried enzyme was added into the reaction at different concentration (10, 20 and 30% (w/v of lactic acid)). The reaction mixture was performed at 60°C for 8 hours using polymerization process as shown above.

4.4 Analytical method

The molecular weight of polymerized PDLLA was obtained by HPLC system using Gel Permeation Chromatography column (TSK-GEL H_{XL} Series with a mobile phase of THF, a flow rate of 1ml/min and a temperature of 30°C. The peak of each molecular mass was detected by using UV system (254 nm) with Polystyrene was used as molecular mass with a range of 269- 9320 Da standard.

4.5 Re-polymerization of PDLLA

The PDLLA degraded products were dissolved in toluene at a concentration of 450 mg/mL and heated to 60°C, and then 10% (w/v) of the freeze dried enzyme was added to the solution. The reaction mixture was continuously stirred at optimum condition from the experiment 4.2 under a nitrogen atmosphere. After that reaction, toluene was removed by evaporation at 60°C and high pressure at 700 mmHg. The PDLLA product was extracted by adding 50 mL of dichloromethane, shook at 150 rpm for 1 hours, the dichloromethane phase was transferred to a fresh flask and dichloromethane was removed by evaporation at 60°C and high pressure at 400 mmHg. The precipitate at the bottom of evaporation flask was dissolved with tetrahydrofuran (THF). The polymerized PDLLA product was used to determination the molecular weight of the polymer section.

CHAPTER 4
RESULT

1. Optimization of PDLLA-degrading enzyme production by *Actinomadura keratinilytica* strain T16-1

1.1 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. 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 days of cultivation as shown in Figure 10. Among these supports, the results of free cells and scrub pad were not significantly different. 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 hours, the bacterial cells grew on the surface and inside the pores of the scrub pad as shown in Figure 11. The small mycelia of strain T16-1 were spread inside and attached well to the scrub pad.

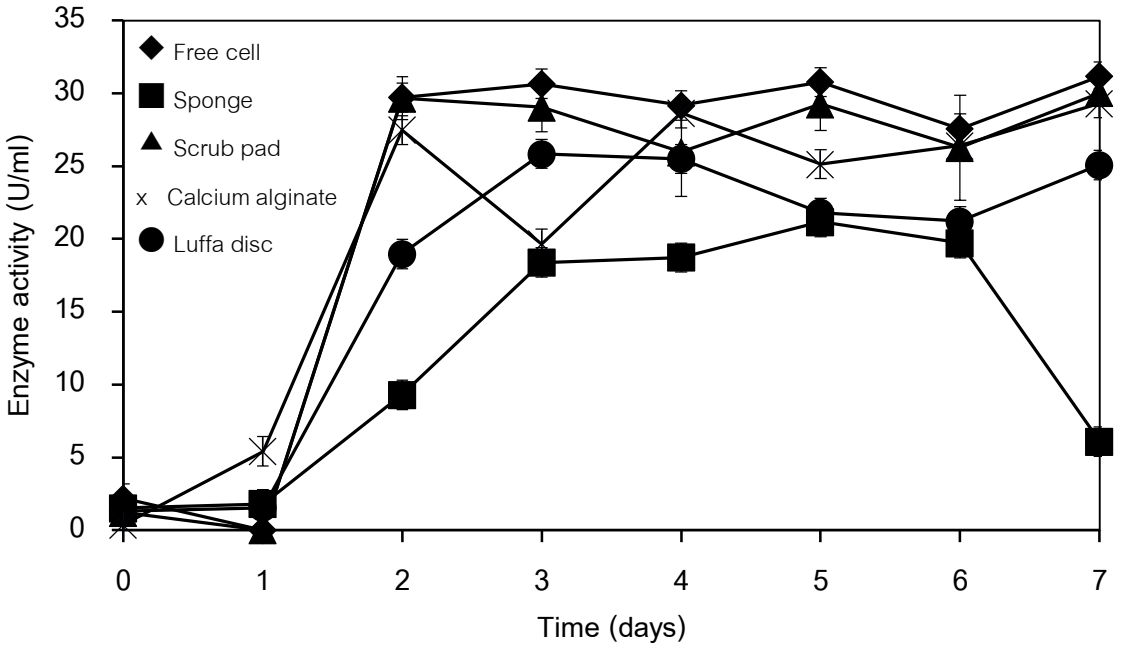


FIGURE 10 Effect of various immobilizers on PDLLA-degrading enzyme production by *A. keratinilytica* strain T16-1.

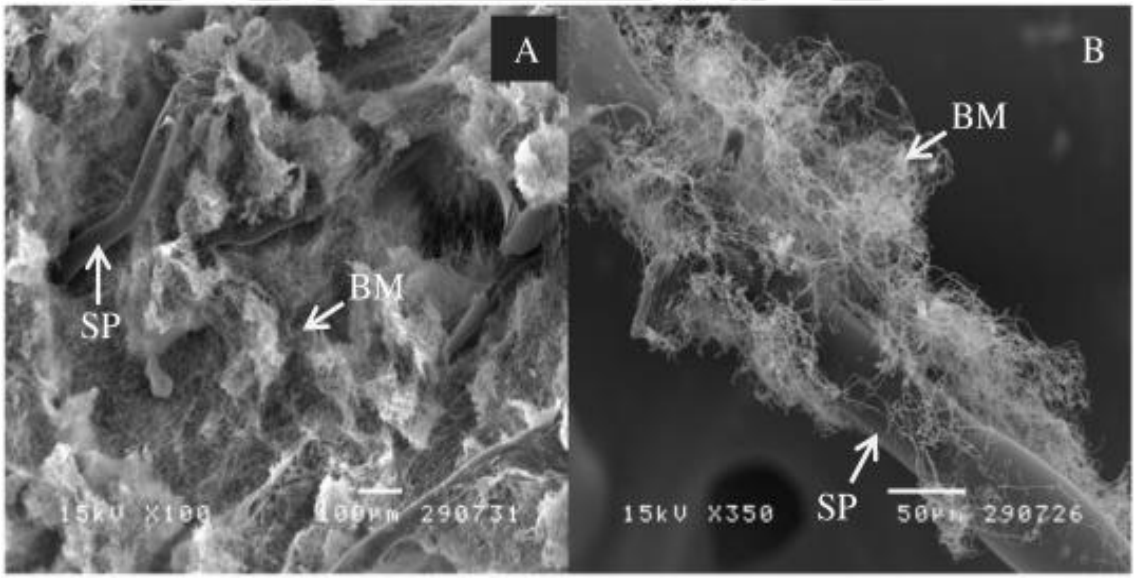


FIGURE 11 Scanning electron micrograph of immobilized strain T16-1 on scrub pad after 48-hours cultivation.

(A) magnification of 100 × and (B) magnification of 350 ×. SP: Scrub pad and BM: Bacterial mycelium.

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

From the results of cell immobilization, a scrub pad was chosen and 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 as shown in Figure 12A. Figure 12B 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 547.38U/g substrate were achieved under optimized conditions.

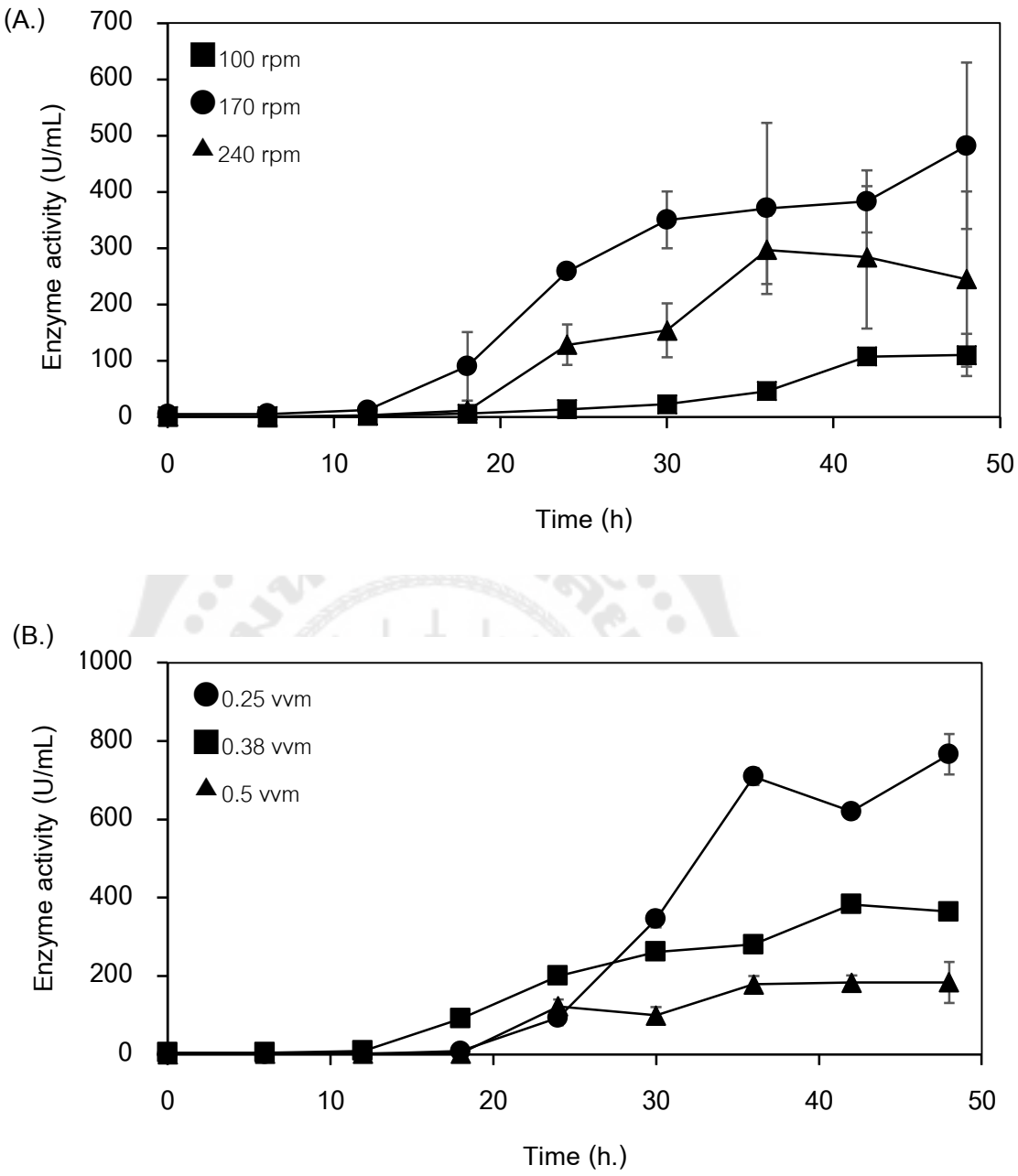


FIGURE 12 Effect of various (A.) agitation speed and (B.) aeration rate on PDLLA-degrading enzyme production by *A. keratinilytica* strain T16-1 in a 5-L stirrer fermenter under batch fermentation.

1.3 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 at the 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. This also helped to retain the cell mass when being used for long period of time or repeated fermentation. In this research, four successive cycles of enzyme production were performed as shown in Figure 13. In each cycle, high enzyme production was detected at 952, 1,095.34, 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 10).

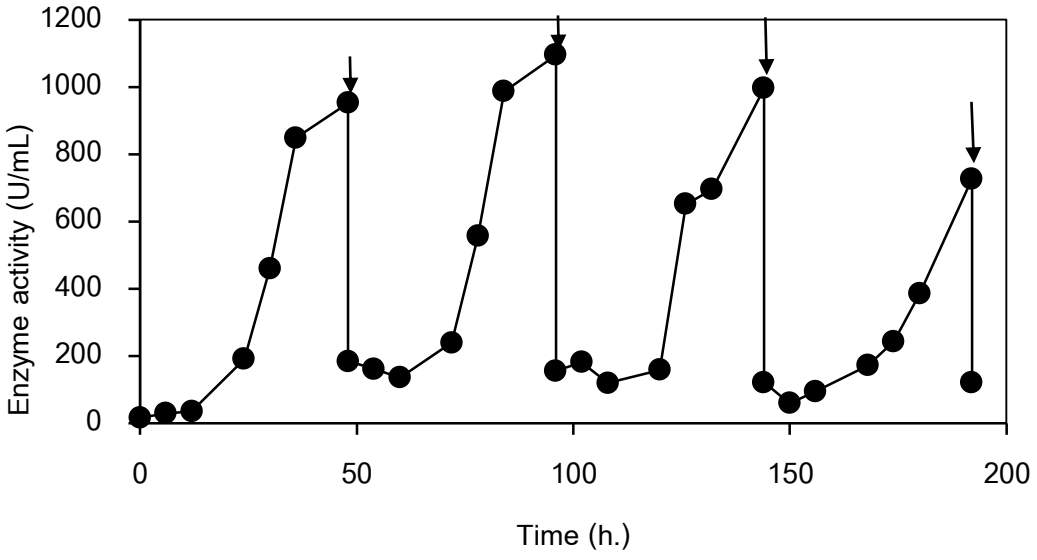


FIGURE 13 PDLLA-degrading enzyme production by *A. keratinilytica* strain T16-1 in a 5-L stirrer fermenter under repeated-batch fermentation at aeration rate of 0.25 vvm, agitation speed of 170 rpm, and temperature of 45°C.

1.4 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. 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 hours, 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. 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 (Table 10).

1.5 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 hours at 45°C, with an agitation speed of 0.25 vvm and an aeration rate of 170 rpm (Figure 14). 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 a10-L fermenter was lower than that in a 5-L fermenter (Table 10).

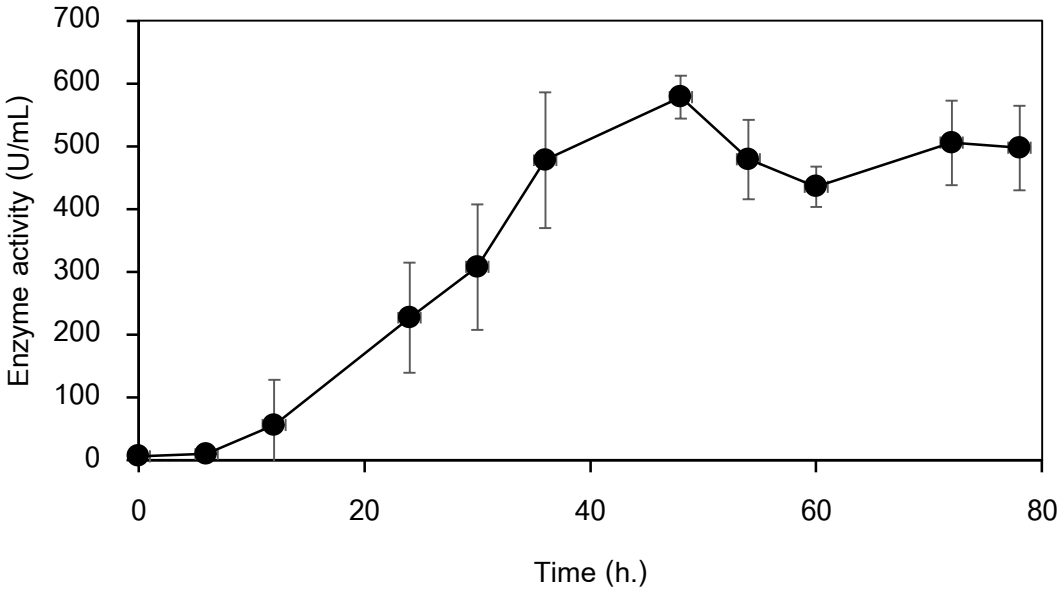


FIGURE 14 PDLLA-degrading enzyme production by *A. keratinilytica* strain T16-1 in a 10-L stirrer fermenter under batch fermentation.

TABLE 10 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 and 10-L stirrer fermenter.

Fermentation scale	Fermentation process	Parameters (mean ± SD)			Time (hours)
		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
5L	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]	-
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 $p < 0.05$.

* showed the average values from four cycles

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

2.1 Factors affecting PDLLA-degradation by a crude enzyme produced by *A. keratinilytica* strain T16-1 in a 5-L stirrer bioreactor

In this work, we focused on the scale-up of PDLLA degradation in a 5-L bioreactor. The result showed that the PDLLA degradation at agitation speeds of 50 and 100 rpm demonstrated lactic acid concentrations of 4,627 and 2,679 mg/L, respectively, at 60ºC after incubation for 60 hours. The percentage of conversion efficiency at agitation speeds of 50 and 100 rpm was 25 and 14, respectively, as shown in Figure 15. The lactic acid concentration at an agitation of 50 rpm was higher than that of 100 rpm by approximately 1.7-fold.

The effect of pH on the PDLLA degradation efficiency was determined. The result showed that PLA degradation at different pH levels (8.0, 9.0 and 10.0) released the lactic acid concentration as 16,651, 9,092 and 7,252 mg/L, respectively, and the percentage conversion were 89, 39 and 48, respectively, at a 50 rpm agitation rate and 60ºC as shown in Figure 16. The highest PDLLA conversion efficiency was obtained at pH 8.0 after a 72 hours reaction time. The lactic concentration at pH 8.0 was higher by approximately 2.3 and 1.8 times at pH 9.0 and 10.0, respectively.

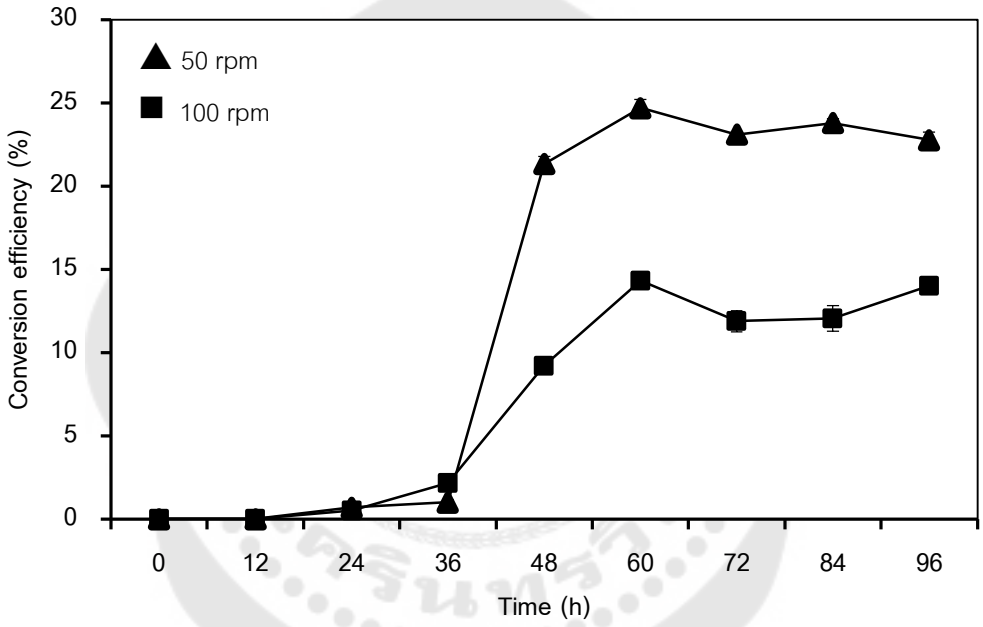


FIGURE 15 The effect of agitation speed on PDLLA degradation by enzyme produced by *Actinomadura keratinilytica* strain T16-1 in 5L stirrer bioreactor under batch condition at pH 9.0 and 60ºC.

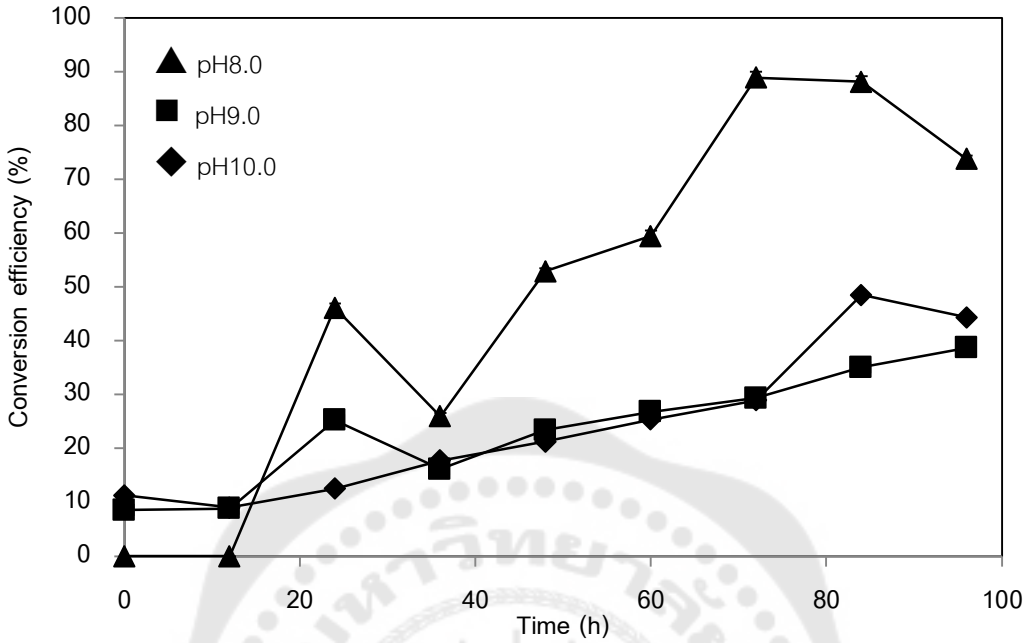


FIGURE 16 The effect of controlled pH on PDLLA degradation by enzyme produced by *Actinomadura keratinilytica* strain T16-1 in 5L stirrer bioreactor under batch condition at 50 rpm of agitation speed and controlled temperature at 60°C.

2.2 Effect of the lactic acid concentration on PDLLA degradation

The effect of lactic acid on the PDLLA degradation was studied at different concentrations (0-8,000 mg/L). The initial pH after adding lactic acid at different concentrations (0-8,000 mg/L) were 9.0, 7.43, 6.85, 4.12 and 3.80, respectively. The result showed the percentage conversion efficiency of 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 Figure 17.

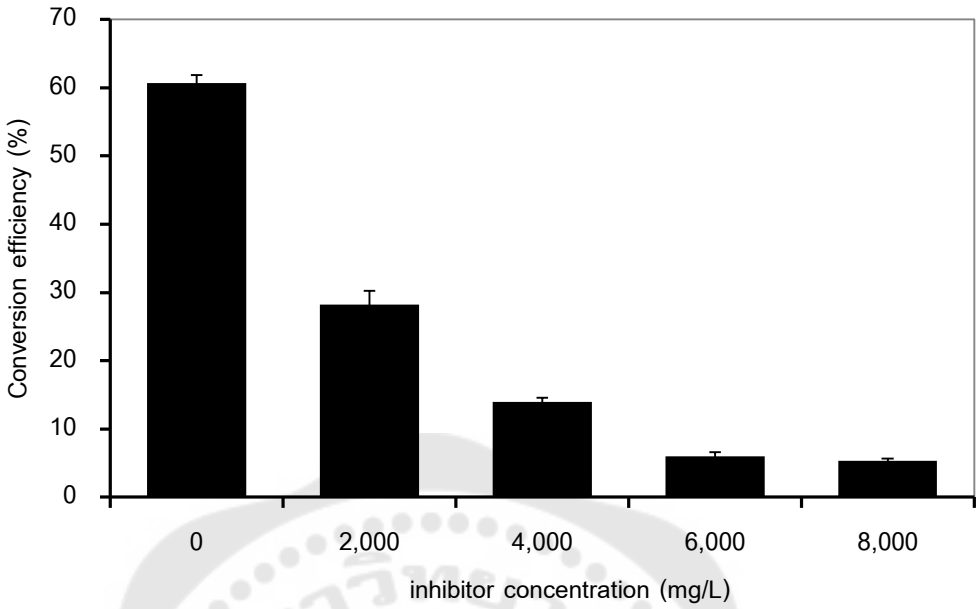


FIGURE 17 The effect of lactic acid concentration as degradation inhibitor on PDLLA degradation at 60°C after incubation for 24 hours.

2.3 Evaluation of the degradation method for the elimination of lactic acid during PDLLA degradation

The method for elimination of lactic acid during PDLLA degradation was studied by the dialysis technique. Using different methods, the result indicated that the percentage of the conversion efficiency were 62, 66 and 99 and the percentage of PDLLA powder weight loss were 14.75, 25.95 and 99.93% from method A, B and C, respectively (method A: control experiment, method B: separated PDLLA degradation and dialysis and method C: simultaneous PDLLA degradation and dialysis) as shown in Figure 18. The highest percentage conversion efficiency and weight loss were obtained after incubation in the reaction mixture for 24 and 48 hours, respectively, and by using method C. Therefore, the most effective method for lactic acid elimination was method C with simultaneous PDLLA degradation and the dialysis method. The lactic concentration of experiment C with 18,018 mg/L was higher than those of experiments A (11,685 mg/L) and B (12,318 mg/L) by approximately 1.54- and 1.46-fold, respectively. Figure 19 showed the residual amount of PDLLA powder after degradation by the crude enzyme

produced by *A. keratinilytica* strain T16-1 through different method. It presents the lowest residual PDLLA powder in the condition of simultaneous PDLLA degradation and dialysis (method C).

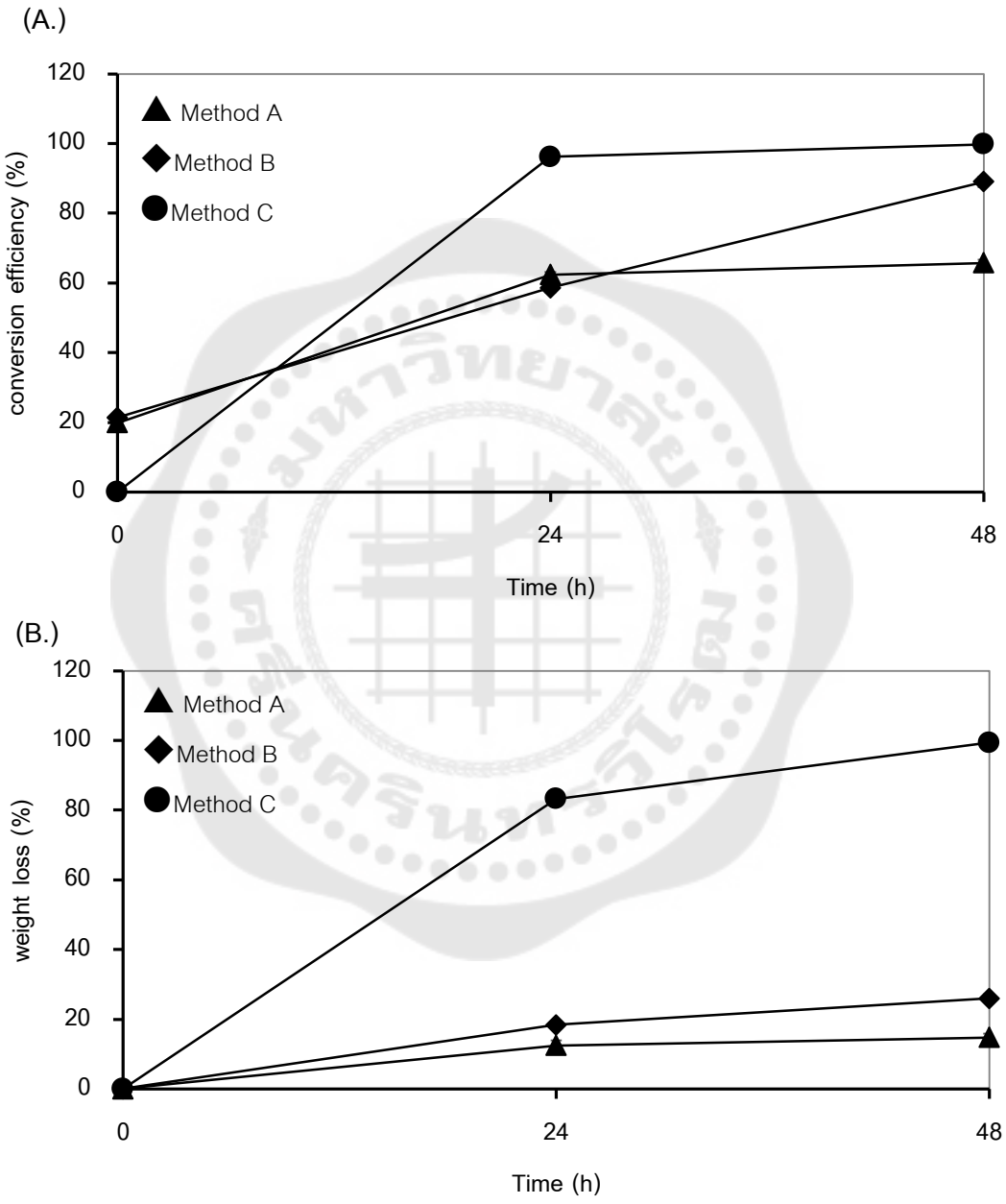


FIGURE 18 Time course of PDLLA degradation by using crude PDLLA-degrading enzyme produced by *Actinomadura keratinilytica* strain T16-1 at different lactic acid elimination methods. (A.) Percentage of conversion efficiency (B.) Percentage of weight loss.

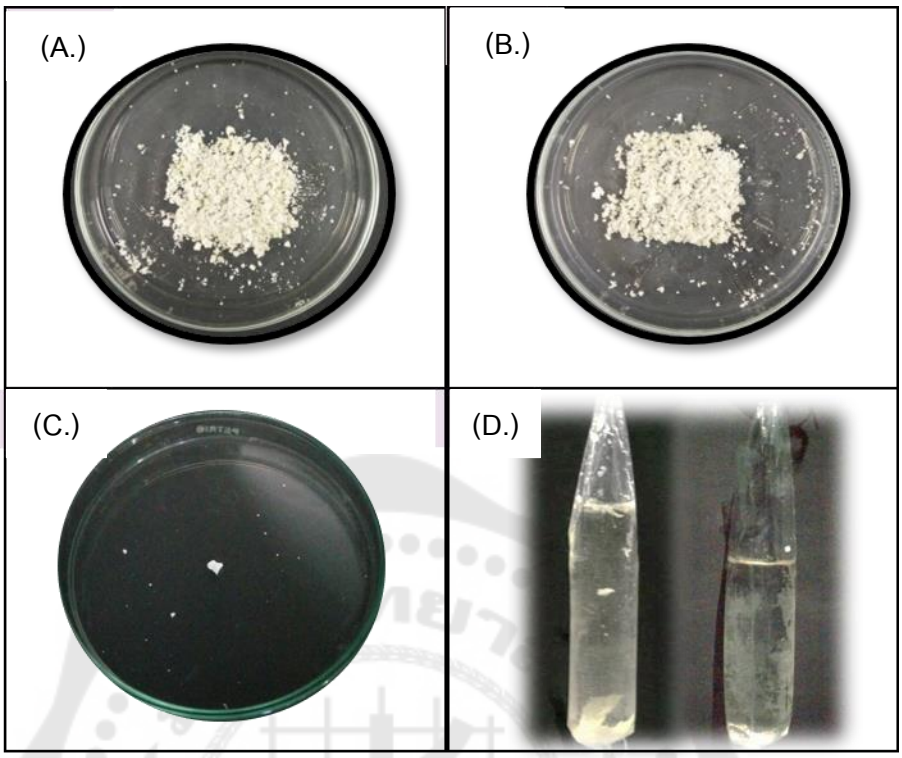


FIGURE 19 The residual amount of PDLLA powder after degradation by the enzyme produced by *A. keratinilytica* strain T16-1 using different method.
(A.) Method A. Control experiment, (B.) Method B. separated PDLLA degradation and dialysis, (C.) Method C. simultaneous PDLLA degradation and dialysis and (D.) PDLLA powder in dialysis bag after 24 hours and 48 hours, respectively

2.4 Commercial PLA product degradation by a crude PDLLA-degrading enzyme

Commercial PDLLA product degradation such as textile, tray, glass, glass lid and straw were evaluated by a crude PDLLA -degrading enzyme produced by *A. keratinilytica* strain T16-1. Table 11 summarizes the percentage of weight loss of PDLLA powder and commercial PLA material degradation by the crude enzyme. The results revealed that the PDLLA textile had higher percentage of weight loss when it was degraded by a simultaneous degradation and dialysis method with 85.36% weight loss. Whereas, PDLLA tray and PDLLA glass lid were degraded with 32 and 31.45% weight loss, respectively. Meanwhile, the controlled experiment showed only 5% weight loss.

TABLE 11 Comparison of percent PDLLA weight loss of PDLLA powder and commercial PDLLA material degradation by using crude PDLLA -degrading enzyme from the strain T16-1 with/without simultaneous degradation and dialysis.

Commercial PDLLA product	Weight loss (g)	Weight loss (%)
Powder	0.993	99.3
Tray	0.32	32.00
Textile	0.089	85.36
Spoon	0.0367	6.88
Glass	0.018	16.60
Straw	0.0041	3.64
Glass lid	0.034	31.45

3. Thermo-solvent tolerant lipase for PDLLA polymerization

3.1 Isolation of solvent tolerant lipase producing actinomycetes for PDLLA re-polymerization

A total of 131 bacterial strain were isolated from 77 soil samples which were collected from Sakerat Biospere Researves, Thailand. Among these strains, 63 isolates were actinomycetes. All actinomycetes were screened for the ability to produce lipase via plating method on tween 80 agar. The 23 isolates of actinomycetes had the precipitation zone on tween 80 agar with colony diameter more than 5.0 mm. as shown in Table 12. Therefore, all isolates were selected and used for further study.

TABLE 12 The isolates of lipase producing actinomycetes and diameter of precipitation zone on tween 80 agar.

Isolate	Diameter of precipitation zone (mm.)	Isolate	Diameter of precipitation zone (mm.)
A16502	6.0	A3802	6.6
A17503	6.3	A3803	5.0
A17504	6.3	A3805	7.2
A19503	6.6	A4101	5.1
A20502	5.0	A4502	9.2
A29402	5.5	A4503	5.2
A3102	6.1	A4601	6.2
A3104	8.6	A4603	6.4
A3105	6.4	A601	6.0
A3202	7.0	A7601	6.0
A3301	6.5	A79401	5.0
A3701	7.0		

3.2 Screening of thermo-solvent tolerant lipase producing actinomycetes

Twenty-three isolates of actinomycetes were investigated for the ability to produce thermo solvent tolerant lipase. The lipase production was performed in production medium. The result showed only 11 isolates had ability for lipase production with high activity between 60-108 U/mL as shown in Figure 20. Subsequently, all 11 isolates were tested for screening of thermo-solvent tolerant lipase production. The enzyme production was performed in production medium containing 10% (v/v) organic solvent (toluene or hexane: chloroform (2:1)). Moreover, the growth of solvent tolerant actinomycetes were investigated by dropping the culture on ISP-2 agar after cultivation. The result showed that the best isolate for thermo-solvent tolerant lipase production was A3301. The isolate A3301 produced the maximum lipase activity of 108 U/mL in

production medium as shown in Figure 20. Besides, the isolate A3301 produced the enzyme with activity of 60 and 80 U/mL in production medium containing 10% toluene and hexane:chloroform, respectively (Figure 21). It could grew well on ISP-2 agar with toluene and hexane:chloroform equal to positive control as shown in Table 13. Subsequently, the thermostability of lipase produced by actinomycetes isolates were performed by incubating the enzyme at different temperature between 37-65°C for 1 hours. The result showed the isolate A3301 has the highest lipase activity of 90 U/mL at temperature 45-55°C and decreased the activity after increasing temperature from 65°C to 80 U/mL as shown in Figure 22. Thus, isolate A3301 was selected based on the ability to produce high thermo-solvent tolerant lipase activity and used for further study.

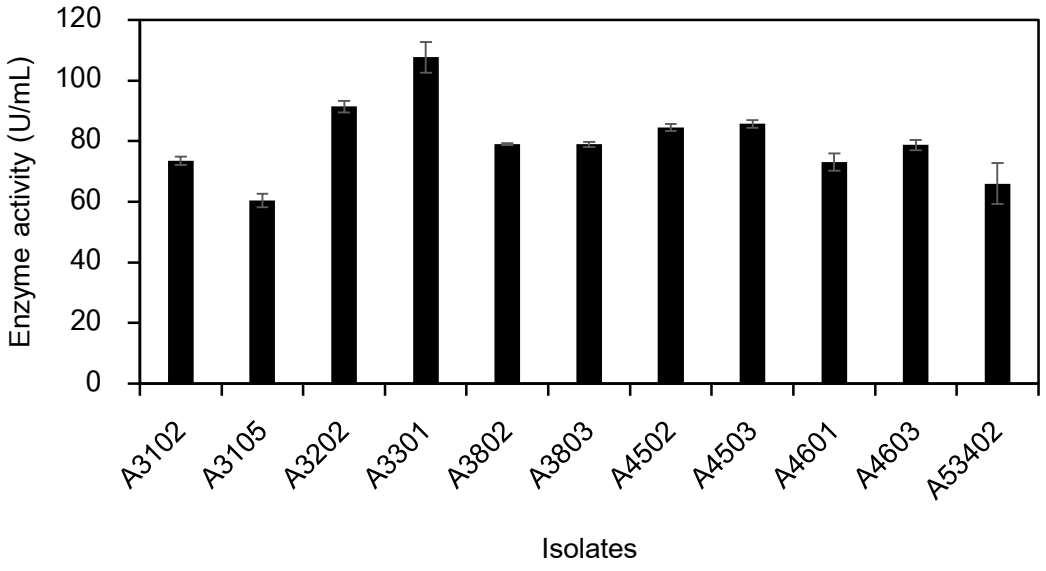


FIGURE 20 The lipase activity of isolated actinomycetes in 50 mL of production medium and incubated at 30°C, 150 rpm for 3 days.

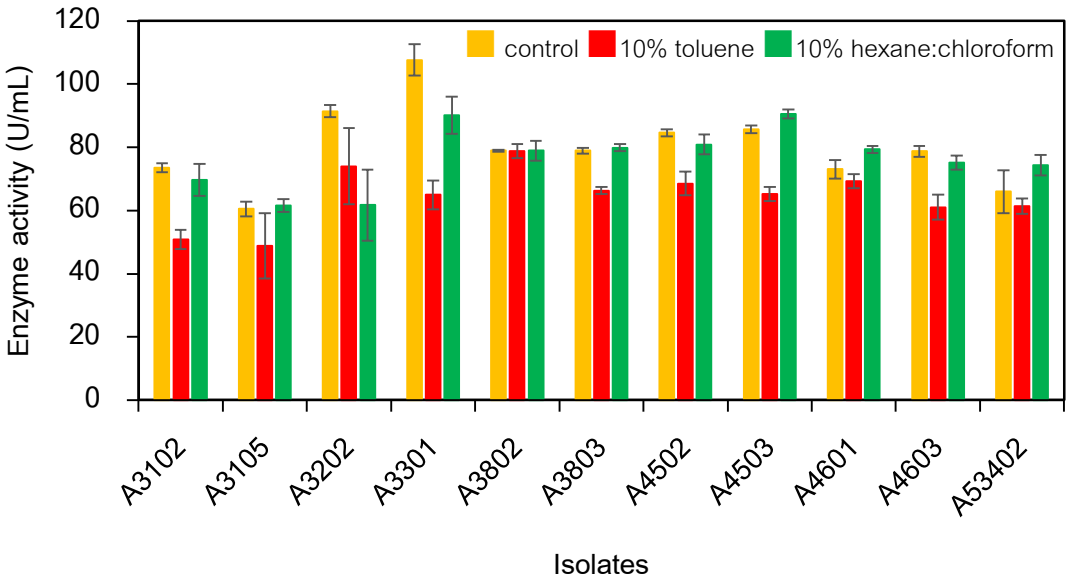


FIGURE 21 The lipase activity of isolated actinomycetes. Lipase production was performed in production media containing 10% organic solvent 50 mL of production media incubated at 30°C, 150 rpm for 3 day.

TABLE 13 Growth of 11 isolates in 10% at different organic solvents

Isolate	10% Toluene	10% Hexane:Chloroform
A3102	+++	+++
A3105	+++	+
A3202	+++	++
A3301	+++	+++
A3802	+++	+++
A3803	+++	+++
A4502	+++	+++
A4503	+++	+++
A4601	+++	+++
A4603	+++	+++
A53402	++	+++

The result was recorded as follow: + doubtfully growth, ++ positive growth and +++ strongly positive growth.

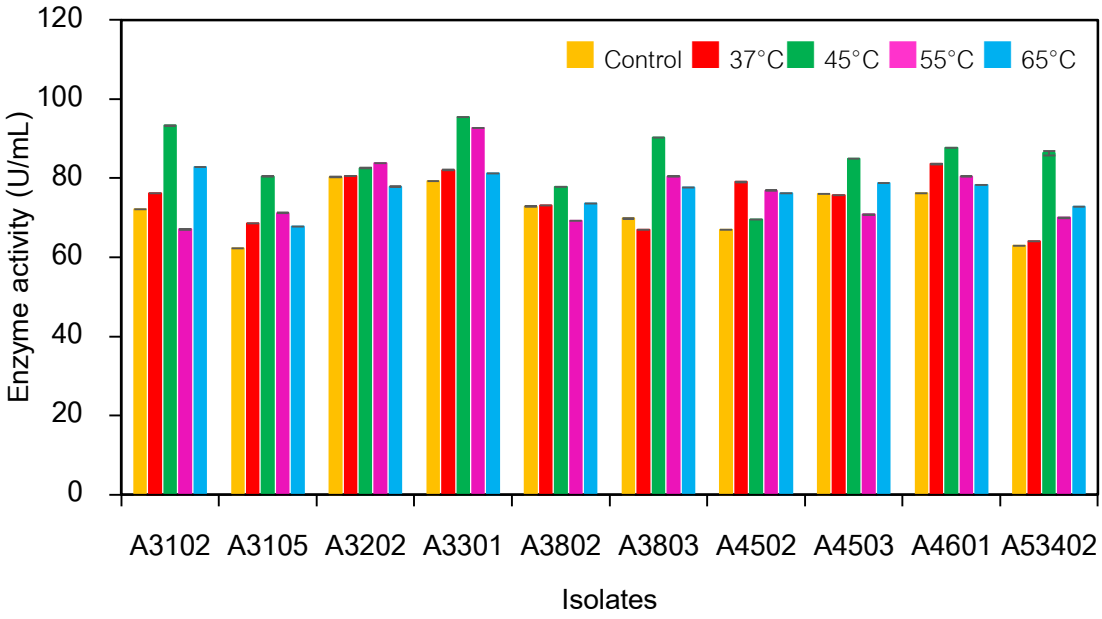


FIGURE 22 The thermostability of lipase produced by isolated actinomycetes.

3.3 Screening of thermo-solvent tolerant lipase

The lipase produced by isolate A3301 showed solvent tolerant and thermostability. The solvent tolerant was performed by incubating the enzyme solution with 10%-20% of organic solvents (toluene and hexane:chloroform) . The result demonstrated that the enzyme produced by isolate A3301 had ability for solvent tolerant at 10-20% of toluene and hexane:chloroform with the enzyme activity of 70 U/mL, 90 U/mL and relative activity with 65% and 83%, respectively as shown in Figure 23.

To test the effect of temperature on stability, the enzyme was pre-incubated at different temperature between 37-65°C. The result showed that lipase produced by isolate A3301 showed thermostability property. The enzyme has high activity at 45 and 65°C with 100 and 88% relative activity, respectively as shown in Figure 24.

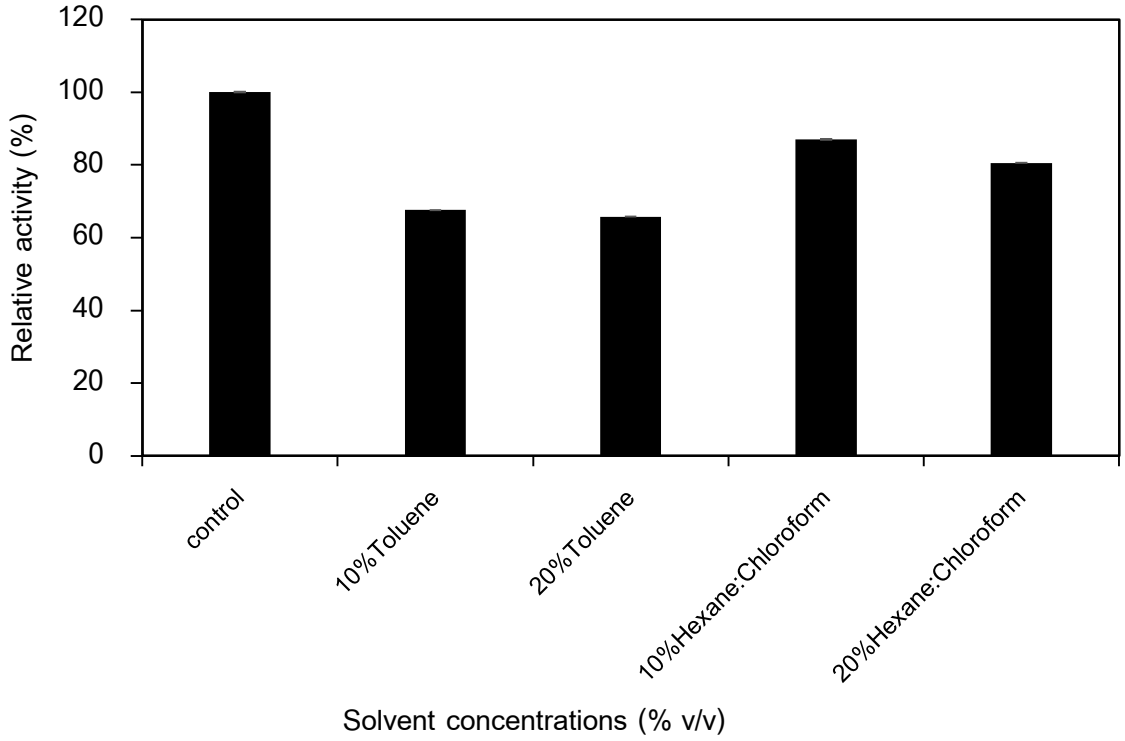


FIGURE 23 The effect of solvent on lipase activity. The lipase produced by isolate A3301 was determined after incubated the enzyme solution with 10-20% v/v of solvent (toluene and hexane:chloroform)

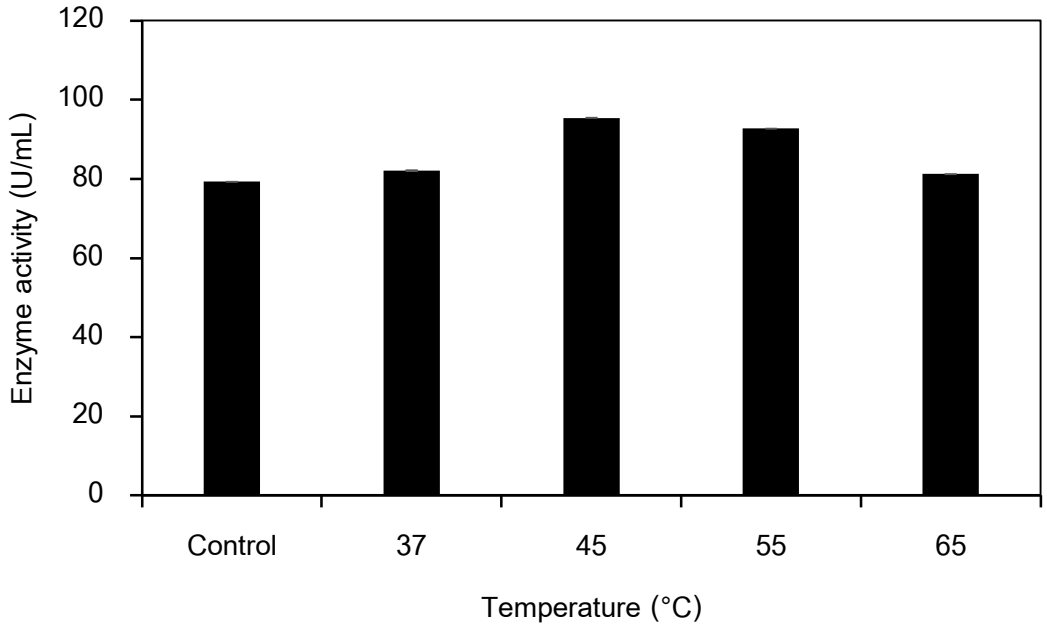


FIGURE 24 The effect of temperature on lipase stability. The lipase of isolate A3301 was incubated at different temperature (37-65°C) for 1 hour before enzyme activity assay.

3.4 Identification of thermo-solvent tolerant lipase producing Actinomycete isolate A3301

The characteristics of the isolate A3301 were shown in Table 14. The colony appeared to be circular and yellow spore on ISP-2 agar. Isolate A3301 was a Gram-positive bacteria, LL-diaminopimelic acid of cell wall type. Color of aerial and substrate mycelium on ISP1-ISP5 were grey-yellow, yellow-yellow, grey-white, white-white and white-yellow, respectively. Spore surfaces were smooth plate. It can utilized lipid, skim-milk and nitrate but not use cellulose and gelatin. It indicated that isolate A3301 is able to produce lipase and protease but cannot produce cellulase and gelatinase. Moreover, it has ability to tolerate NaCl at concentration of 0-7% in ISP2 agar. Isolate A3301 was able to use various carbon source such as dextrose, xylose, mannose, galactose, maltose and lactose.

TABLE 14 Biological characteristics of isolate A3301

Characteristics	Isolate A3301
Morphology	
Gram's stain	Gram-positive
Spore	+
Cell wall type	LL-DAP
Color of mycelium on different medium	
ISP-1	
Aerial mycelium	Grey
Substrate mycelium	Yellow
ISP-2	
Aerial mycelium	Yellow
Substrate mycelium	Yellow
ISP-3	
Aerial mycelium	Grey
Substrate mycelium	White
ISP-4	
Aerial mycelium	White
Substrate mycelium	White
ISP-5	
Aerial mycelium	White
Substrate mycelium	Yellow
Biochemical characterization	
Lipid	+
Skim milk	+
Cellulose	-
Gelatin	-
Nitrate	+

Table 14 (Continued)

Characteristic	Isolate A3301
Enzyme production	
Lipase	+
Protease	+
Cellulase	-
Gelatinase	-
Nitrate reductase	+
NaCl tolerant at 0-7%	+
Utilization of carbon source	
Dextrose	+
Xylose	+
Mannose	+
Galactose	+
Maltose	+
Lactose	+

3.5 Phylogenetic tree analysis

Strain A3301 was identified by 16s rDNA sequencing. The 16s rDNA sequences of ten different *Streptomyces* species obtained from NCBI database were compared. Sequences were analyzed using neighbor-joining method. Strain A3301 belongs to family Streptomycetaceae and identified closely related species *Streptomyces albidoflavus*, *Streptomyces hydrogenans*, *Streptomyces violascens* and *Streptomyces daghestanicus* with 100% similarity, respectively. The phylogenetic relationship of closely related *Streptomyces* was shown in Figure 25

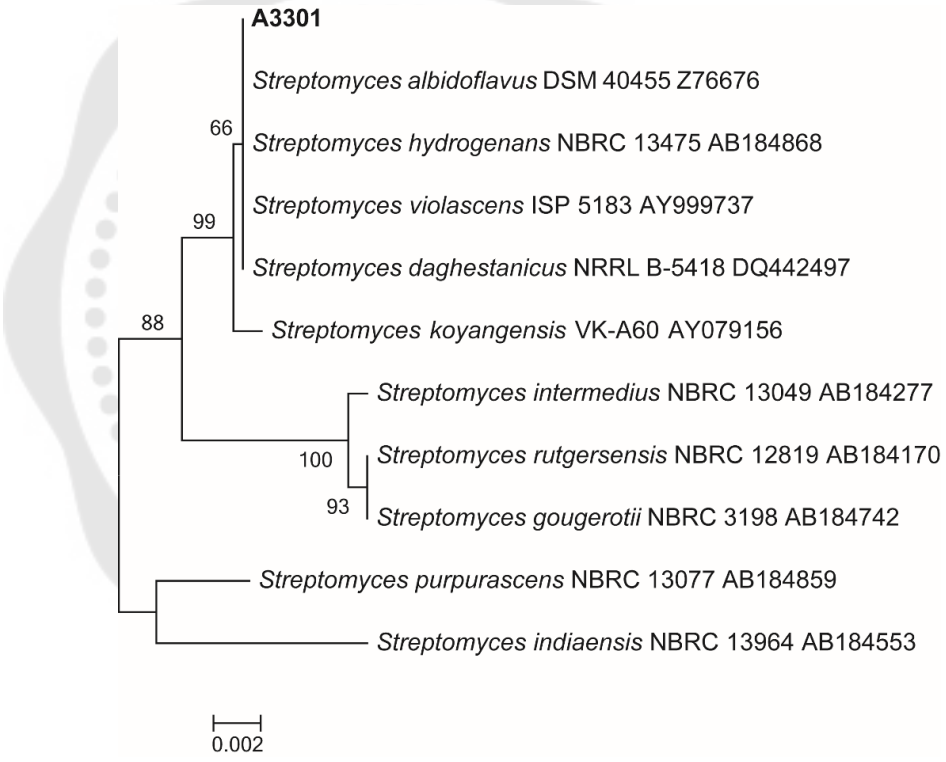


FIGURE 25 Phylogenetic tree showing the relationship of isolate A3301 to other *Streptomyces* species. Phylogenetic tree was constructed by neighbor-joining method. Bootstrap values at branching point and expressed as percentages from 1,000 replication.

3.6 Optimization of thermo-solvent tolerant lipase production by strain A3301

The first experiment, lipase production by strain A3301 was using different medium studied for improving lipase activity. Three medium arrangements (medium A, B and C) were investigated for lipase production. The result showed the lipase activity produced from strain A3301, medium A, B, C and D were 260, 283, 323 and 331 U/mL, respectively (Figure 26). The best production medium for lipase production was medium C which containing 10g/L of oil seed cake. However, the medium supplement with oil seed cake and rice bran oil were not suitable for the enzyme purification for the future study because it has the small particles that make the column clogging. Therefore, the medium A (without oil seed cake and rice bran oil) was selected for subsequent experiments.

After that, the optima condition for lipase production was determined. The first step of optimization is to study the limits of medium components for maximum lipase production. From the results of improving medium, The Medium A, was selected as the best production medium for thermo-solvent tolerant lipase. We investigated the effect of time for 7 days. The maximum lipase production was 160 U/mL after incubation at 30°C, 150 rpm for 3 days as shown in Figure 27. Afterwards, the effect of carbon sources on lipase production was studied. Various carbon sources were investigated including dextrose, xylose, mannose, galactose, maltose and lactose. The highest lipase activity was 306 U/mL in a production medium containing xylose as carbon source as shown in Figure 28. After that, the effect of xylose concentration on lipase production was investigated at 0-3% in production medium. The maximum of lipase production was 313 U/mL at the 1.5% of xylose as shown in Figure 29. Subsequently, the effect of nitrogen sources on lipase activity was studied including malt extract, tryptone, peptone, soy peptone, yeast extract, $NaNO_3$, $(NH_4)_2SO_4$ and KNO_3. Yeast extract was the best nitrogen sources for lipase production with the lipase activity of 240 U/mL. However, this lipase could produce by using the organic nitrogen source such as malt extract, tryptone, peptone, soy peptone and yeast extract. While, the strain A3301 could not use the inorganic nitrogen source such as $NaNo_3$, $(NH_4)_2SO_4$ and KNO_3 for lipase production

as shown in Figure 30. After that, the effect of yeast extract concentration on lipase production was investigated between 0-3% (w/v). The optimum concentration of yeast extract as nitrogen soure for lipase production was 2% (w/v) with lipase activity of 255 U/mL as shown in Figure 31. Subsequently, physicochemical factors (temperature and initial pH) of production medium were studied. The effect of initial pH of production medium was investigated at different initial pH between 5.0-10.0. The maximum of lipase production was 239 U/mL at the initial pH at 7.0 as shown in Figure 32. Moreover, the lipase production was demonstrated at various temperatures between 25°C-40°C. The maximum of lipase production was 321 U/mL when incubated at 30°C (Figure 33).

In conclusion, the optimum condition for lipase production by strain A3301 was 1.5% (w/v) xylose, 2% (w/v) yeast extract at initial pH 7.0, incubated at 30°C, 150 rpm for 3 days. The maximum of lipase production was 321 U/mL, which higher than the primary production medium with approximately 2 folds.

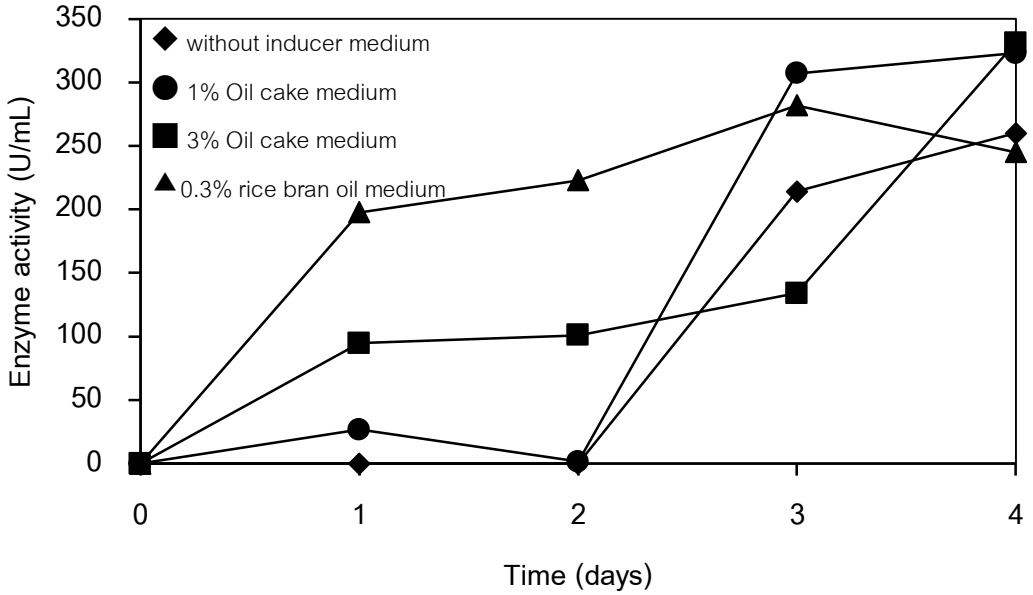


FIGURE 26 Time course of lipase production by strain A3301 with difference medium in 250-mL Erlenmeyer flasks, incubated at 30°C, 150 rpm for 4 days.

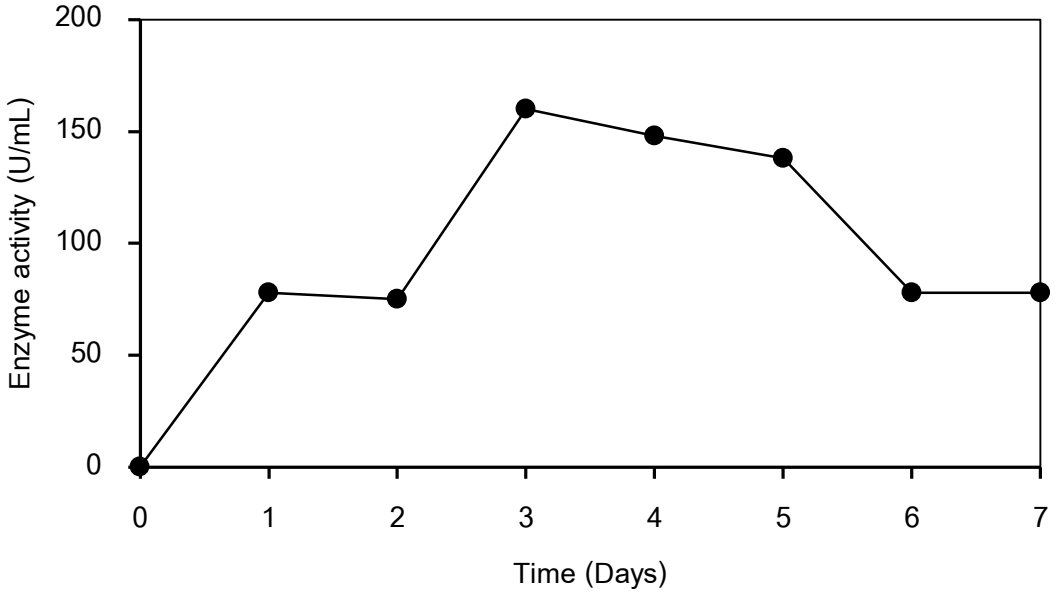


FIGURE 27 Time course of lipase production by strain A3301 in medium A in 250-mL Erlenmeyer flasks, incubated at 30°C, 150 rpm for 7 days.

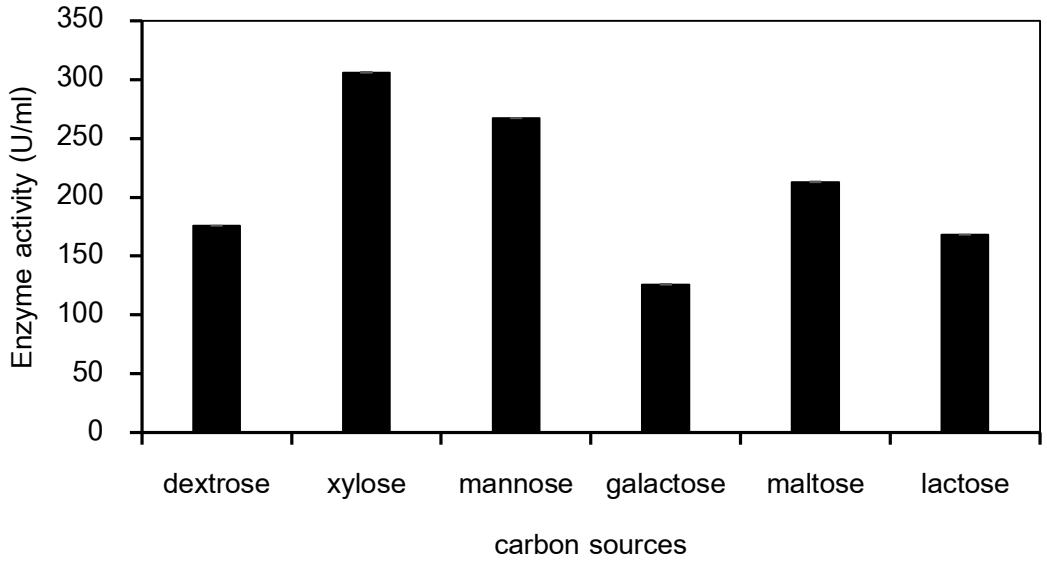


FIGURE 28 The effect of carbon sources on lipase production by strain A3301 in 250-mL Erlenmeyer flasks, incubated at 30°C, 150 rpm for 3 days.

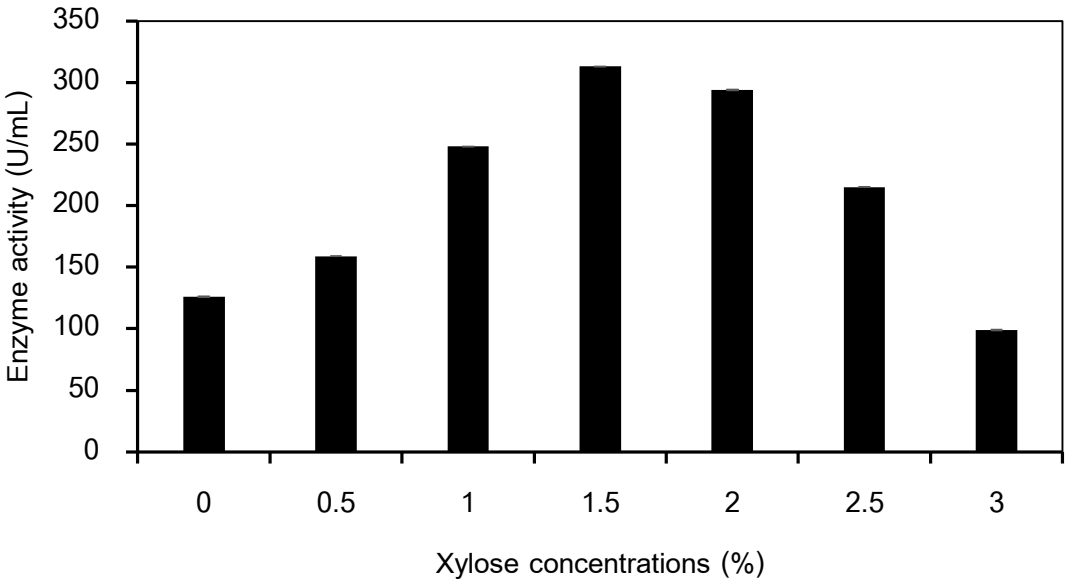


FIGURE 29 The effect of concentration of xylose on lipase production by strain A3301 in 250-mL Erlenmeyer flasks, incubated at 30°C, 150 rpm for 3 days.

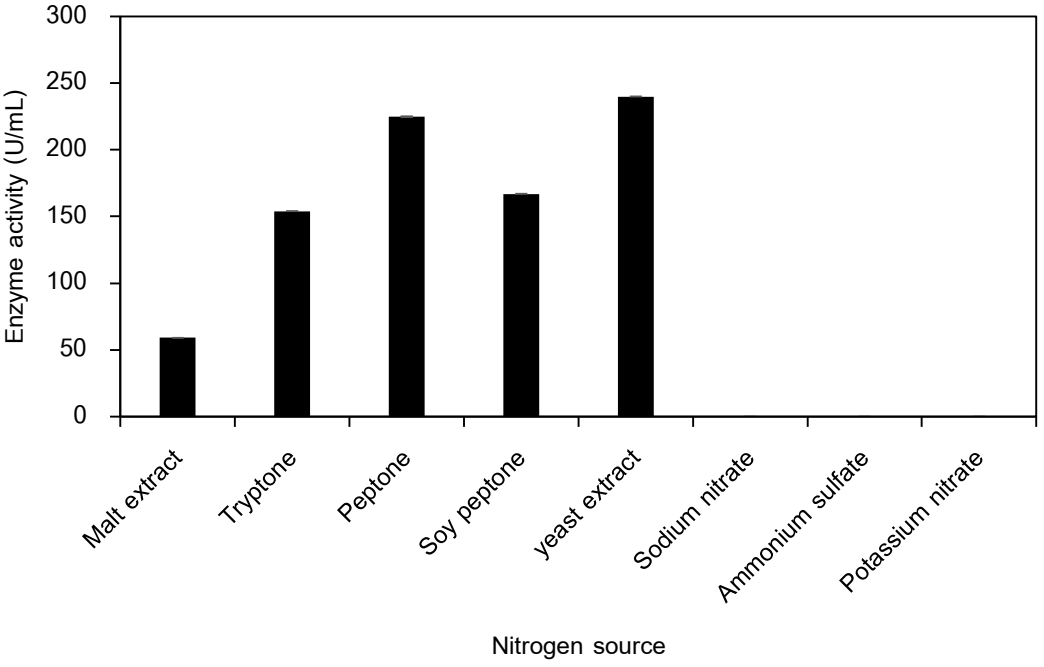


FIGURE 30 The effect of nitrogen sources on lipase production by strain A3301 in 250-mL Erlenmeyer flasks, incubated at 30°C, 150 rpm for 3 days.

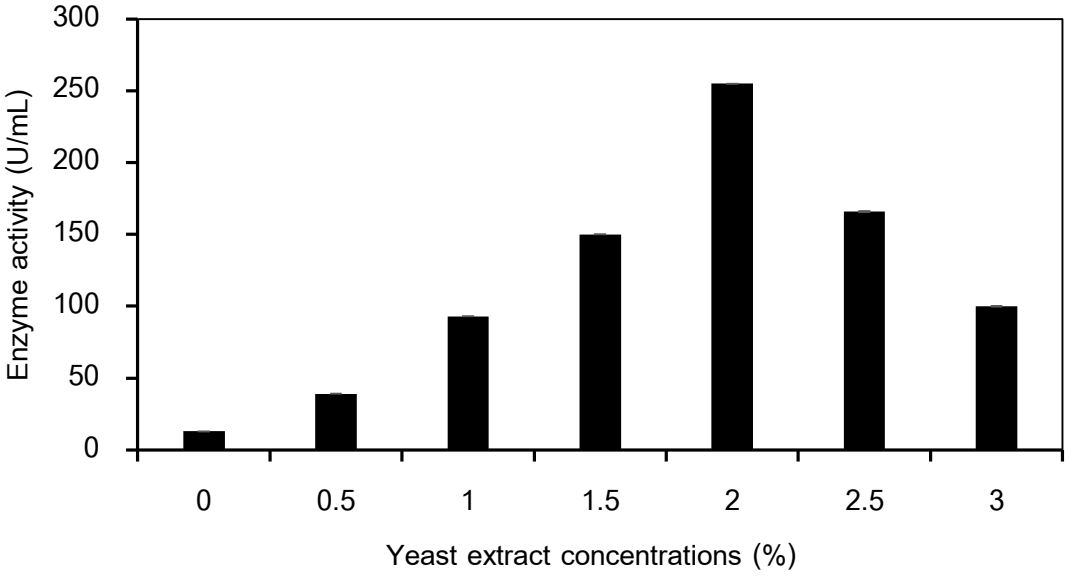


FIGURE 31 The effect of concentration of yeast extract on lipase production by strain A3301 in 250-mL Erlenmeyer flasks, incubated at 30°C, 150 rpm for 3 days.

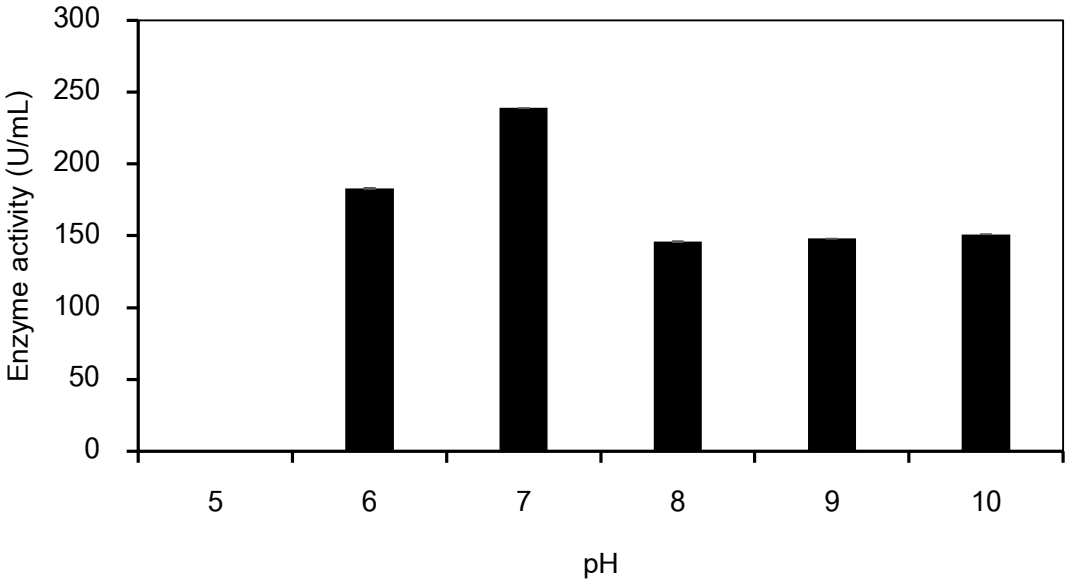


FIGURE 32 The effect of initial pH on lipase production by strain A3301 in 250-mL Erlenmeyer flasks, incubated at 30°C, 150 rpm for 3 days.

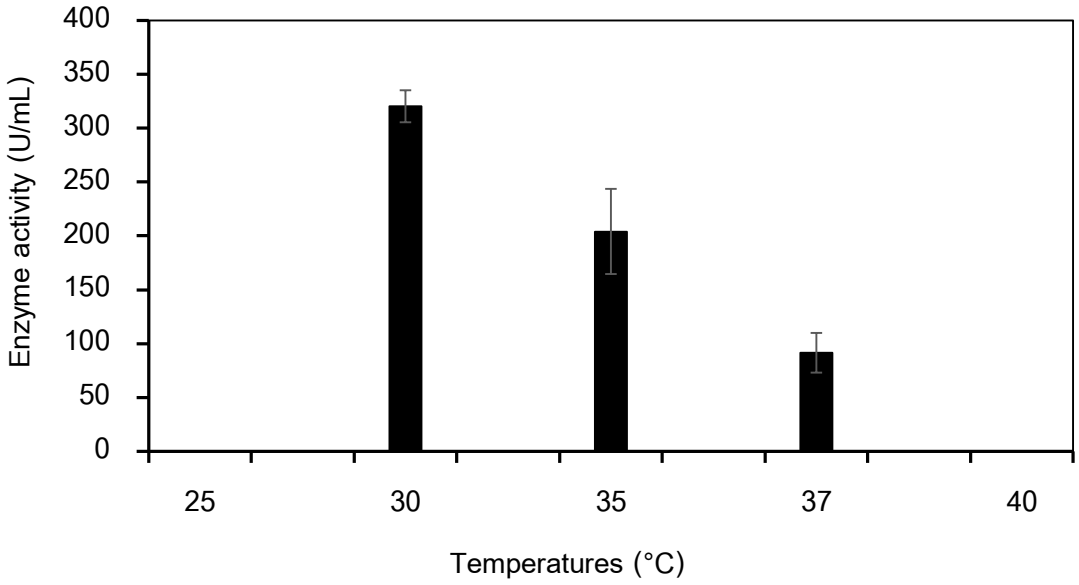


FIGURE 33 The effect of temperature on lipase production by strain A3301 in 250-mL Erlenmeyer flasks, incubated at 30°C, 150 rpm for 3 days.

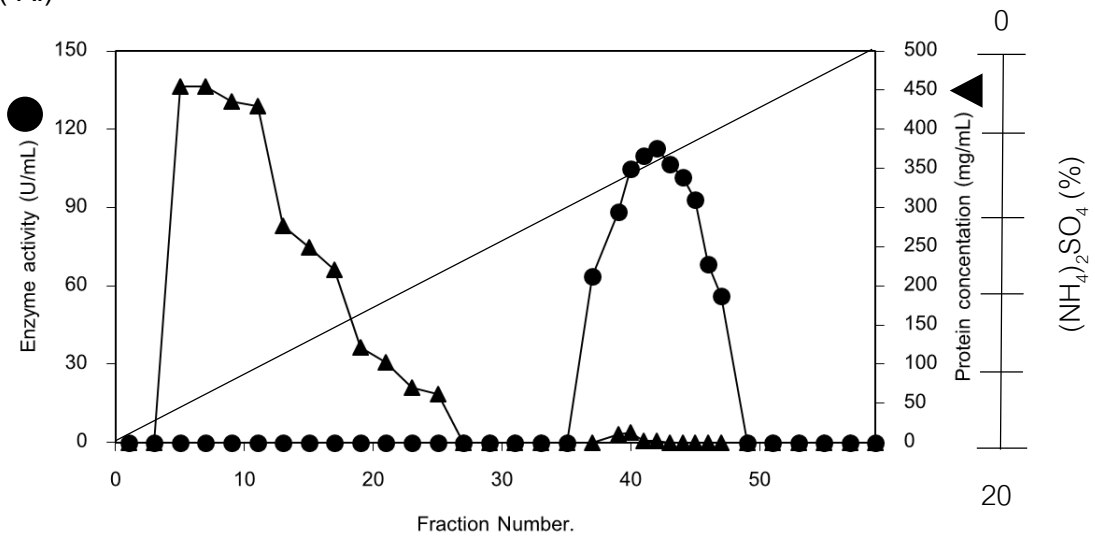
3.7 Purification of thermo-solvent tolerant lipase produced by strain A3301

A summary of a purification of thermo-solvent tolerant lipase from the strain A3301 was shown in Table 15. In the first step, the enzyme was purified by Butyl-650M Toyopearl chromatography. The result showed the specific activity of 27,000 U/mg and 544 of purification fold. After that, the active fractions were applied into DEAE-650M chromatography. The specific activity was 5,600 U/mg and 113 of purification fold, which resulted in single peak of purification profile as shown in Figure 34. The purified lipase showed a single band on a 14% SDS-PAGE analysis. The molecular mass was found to be 45 kDa was visualized by Coomessie brilliant blue staining as shown in Figure 35.

TABLE 15 Purification of thermo-solvent tolerant lipase from *Streptomyces* sp. strain A3301.

Purification step	Volume (mL)	Enzyme activity (U/mL)	Total activity (U)	Protein concentration (mg/mL)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Recovery yield (%)
Crude enzyme	300	119	35,700	2.4	720	49.6	1	100
Butyl-650M	100	81	8,100	0.003	0.3	27,000	544	4
DEAE-650M	5	56	280	0.01	0.05	5,600	113	0.78

(A.)



(B.)

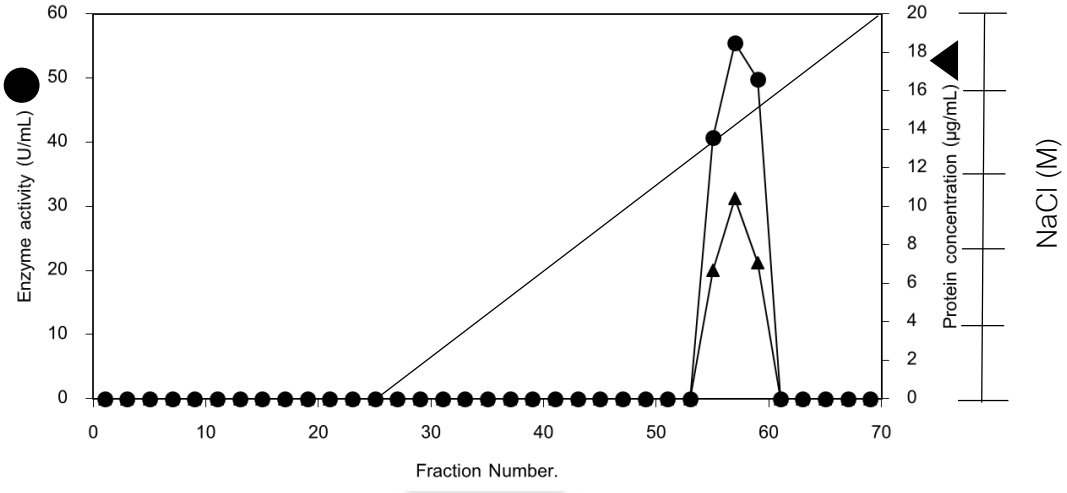


FIGURE 34 Purification profile of thermo-solvent tolerant lipase produced by *Streptomyces* sp. strain A3301 from (A.) Butyl toyopearl 650M hydrophobic chromatography and (B.) DEAE 650 M anion-exchange chromatography.

The enzyme was eluted with Tris-HCl buffer pH 9.0 with gradient 20%-0% of $(NH_4)_2SO_4$ and Tris-HCl buffer pH9.0 with gradient 0-2 M NaCl, respectively. The lipase activity (●) was determined by using p-nitrophenol palmitate as substrate and protein concentration (▲) was determined by Lowry's method

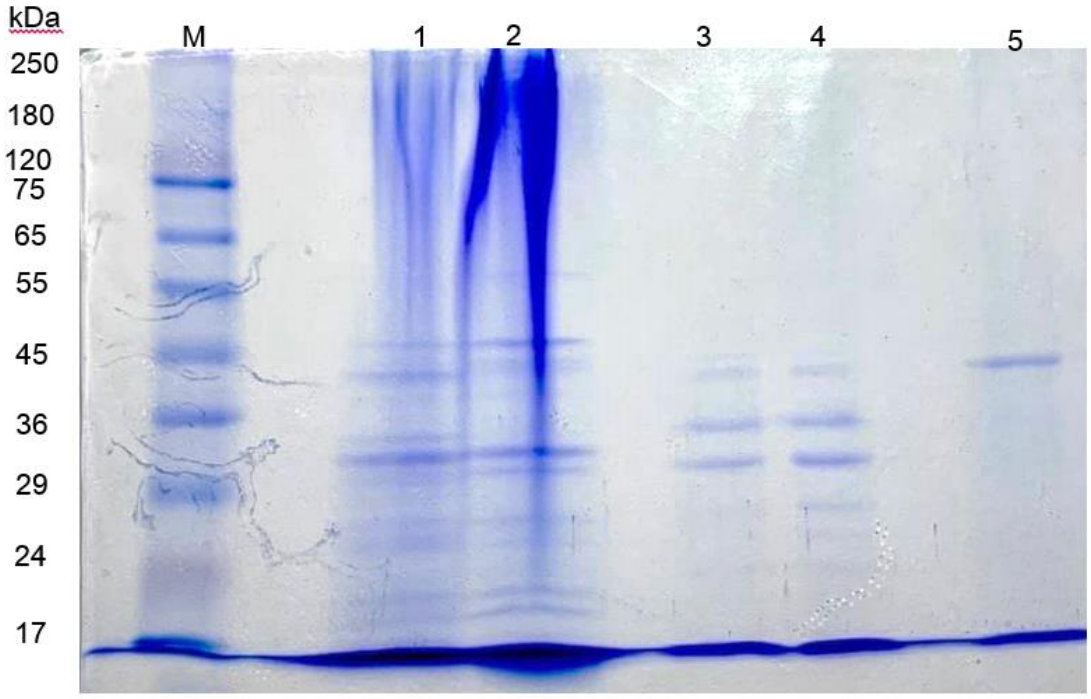


FIGURE 35 SDS-PAGE analysis of purification step of thermo-solvent tolerant lipase from *Streptomyces* sp. strain A3301.

Lane M, molecular marker; Lane 1-2, crude enzyme; Lane 3-4, active fraction from Butyl toyopeal 650M chromatography; Lane 5, active fraction from DEAE toyopeal 650 chromatography.

3.8 Characterization of purified lipase

Lipases are highly diversified in their catalytic properties such as the effect of pH, temperature, metal ions, inhibitors, surfactants and organic solvents on lipase activity. The effect of temperature on lipase activity was studied. The optimum temperature and the thermo-stability of A3301 lipase exhibited at 60°C and 30-55°C, respectively as shown in Figure 36 A, B. The optimum pH of lipase activity was determined. The purified enzyme exhibited the optimum pH at pH 9.0 and the stable at a pH range of 8.0-9.0 (Figure 37 A, B). A summary of the effect of organic solvents on lipase activity was demonstrated in Table 16. The purified enzyme activity was inhibited by 1-butanol, isopropanol, methanol, ethanol, acetonitrite and ethyl acetate with relative activity of 10, 44, 35, 40, 46 and 8%, respectively. While, the organic solvents had no effect on lipase activity of crude enzyme. In addition, DMSO were found to enhance the enzyme activity with relative activity of 147%. The effect of 1mM of metal ions, all of metal ions were promoted the enzyme activity but only Ba^{2+} ion inhibited the enzyme activity of purified enzyme as shown in Table 17. The effect of 1mM of inhibitors and 0.05% of surfactant, the purified enzyme was inhibited by tween85 with relative activity of 14%. While, the crude enzyme was inhibited by tween 85, triton-x100 and EDTA of 26, 59 and 78 % relative activity as shown in Table 17.

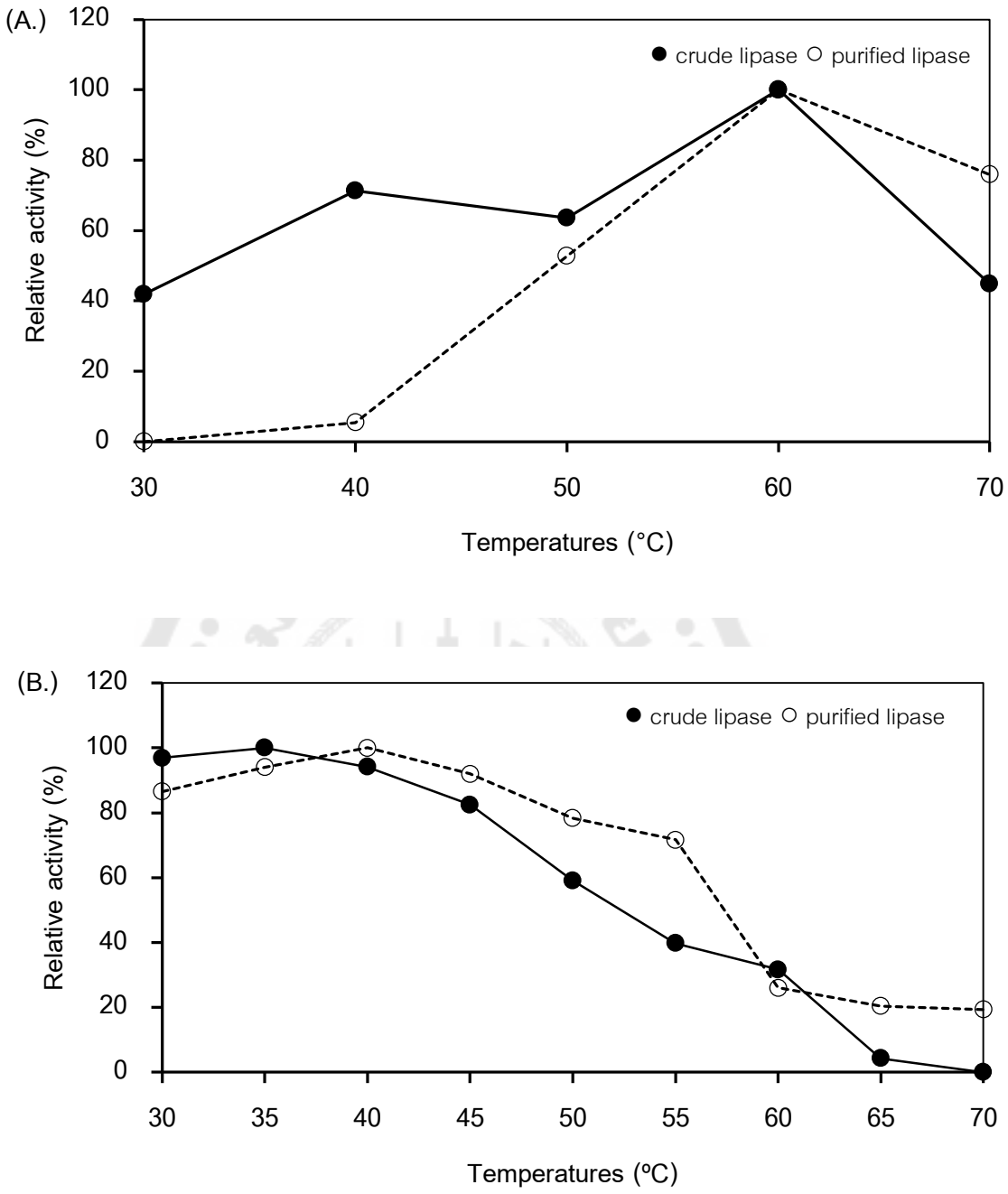


FIGURE 36 The effect of temperature on lipase activity from *Streptomyces* sp. strain A3301. A) optimum temperature and B) thermo-stability of lipase.

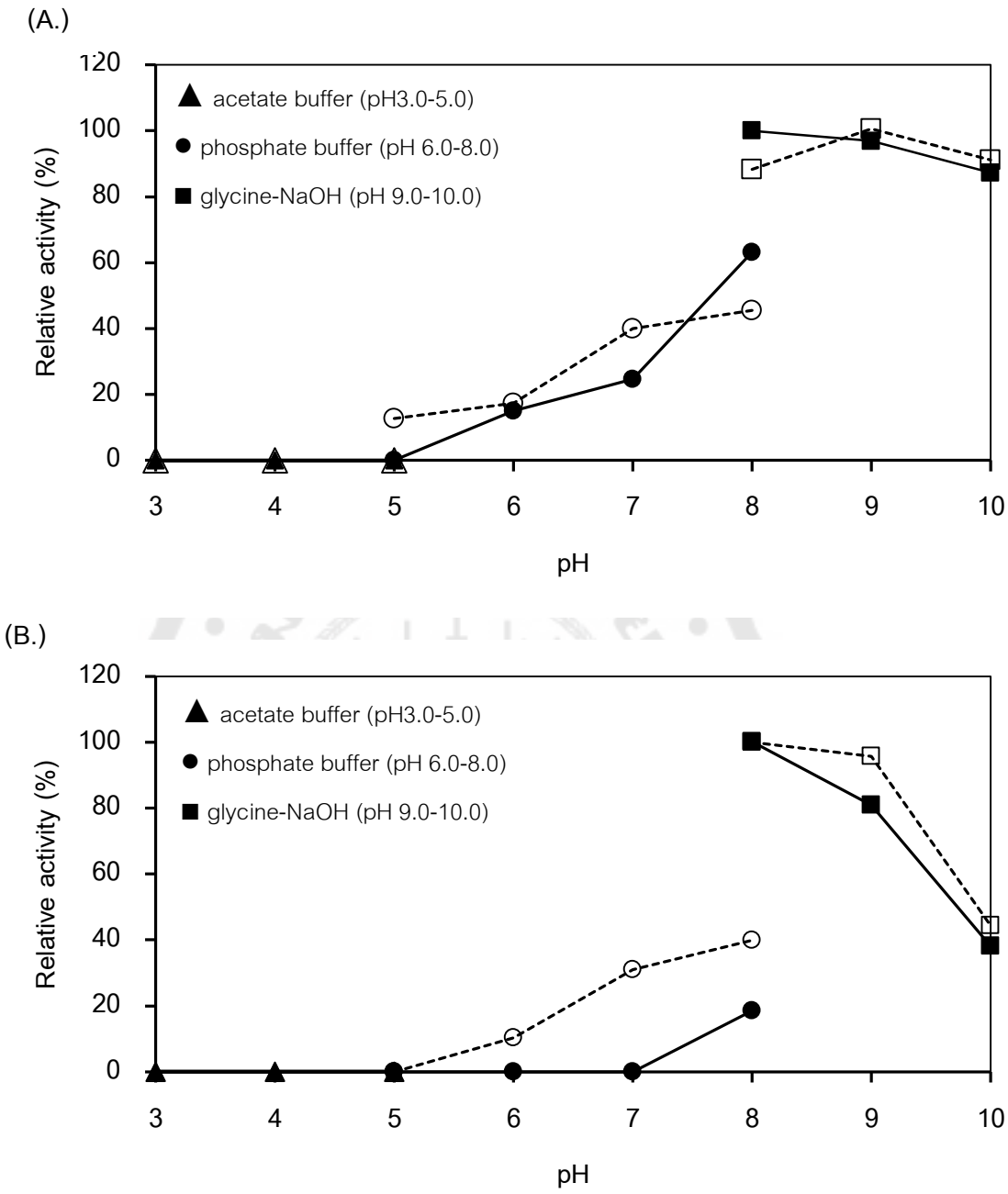


FIGURE 37 Effect of pH on lipase activity from *Streptomyces* sp. strain A3301. (A.) optimum pH and (B.) pH stability of lipase. Crude lipase (close symbols) and purified lipase (open symbols).

TABLE 16 The effect of organic solvent on the A3301 lipase activity.

Organic solvents	Relative activity (%)	
	Crude enzyme	Purified enzyme
Control	100	100
Acetone	178	76
Chloroform	203	63
Toluene	94	68
1-butanol	3	10
Isopropanol	55	44
Methanol	184	35
Ethanol	141	40
Acetonitrite	124	46
Ethyl acetate	202	8
DMSO	168	147

TABLE 17 The effect of metal ions, cheating agent and surfactant on the A3301 lipase activity

Effectors	Relative activity (%)	
	Crude enzyme	Purified enzyme
Control	100	100
1mM of metal ions		
Li^{+}	110	137
Zn^{2+}	116	120
Mg^{2+}	115	101
Ca^{2+}	113	119
Co^{2+}	119	124
Fe^{2+}	98	101
Cu^{2+}	102	123
Ni^{2+}	100	135
Ba^{2+}	118	75
1mM of cheating agent		
EDTA	78	100
0.05% of surfactants		
SDS	98	139
Tween85	26	14
Triton X-100	59	104

4. Re-polymerization of PDLLA by crude thermo-solvent tolerant lipase produced from strain A3301

4.1 Optimization of PDLLA polymerization by crude lipase using lactic acid as monomer

The PDLLA polymerization was performed under condensation system. The commercial lactic acid was mixed with toluene at a final concentration of 450 mg/ml, heated to 60°C, and then 10% (w/v of lactic acid) of the dried enzyme produced by strain A3301 was added into the solution at 10% (w/v of lactic acid). The reaction mixture was continuously stirred at 60°C under a nitrogen atmosphere for 6 hours. The effect of reaction times on PLA polymerization was investigated at various reaction times (8, 16 and 24 hours). The maximum of PDLLA polymerization was achieved at final lactic concentration 450g/L, 60°C for 8 hours of reaction time with the highest PLA molecular weight of 525 Da (n=7). While, the PDLLA polymerization at 16 and 24 hours was decreased of 289 and 242 Da, respectively. After that, various temperatures was studied at 50-70°C. The result showed the PLA polymerization at 60°C was synthesized the heaviest PDLLA molecular weight of 525 Da (n=7) as same as the first condition. The molecular weight of PDLLA was performed at 50 and 70°C with Mw of 249 and 250 Da, respectively. The results indicated that the molecular weight of PDLLA at 50 and 70°C were lower than 60°C about two times. Subsequently, concentration of dried lipase was demonstrated at different of dried lipase concentrations at 10-30% (w/v of lactic acid). The highest molecular mass of PDLLA product was completed at 10% (w/v of lactic acid) of 525 Da (n=7). Moreover, the concentration of dried lipase was increased the PDLLA polymerized decrease. The molecular mass of PDLLA when used dried lipase concentration at 20 and 30% (w/v of lactic acid) were at 238 and 235 Da, respectively. Summary of PDLLA polymerization, the best condition for PDLLA synthesis was achieved at 60°C, dried lipase concentration of 10% (w/v of lactic acid), for 8 hours of reaction time and fixed lactic concentration at 450g/L under nitrogen atmosphere. The highest molecular weight of PDLLA product was 525 Da (n=7).

4.2 Re-polymerization of PDLLA

Re-polymerization of PDLLA was investigated by using PDLLA degraded products from section 2.4 as substrate. The PDLLA degraded product were dissolved in toluene at a concentration of 450 mg/mL and heated to 60°C, with 10% (w/v) of the freeze dried enzyme being added to the solution. The reaction mixture was continuously stirred at optimum condition from the experiment 4.2 under a nitrogen atmosphere. The result showed the re-polymerization process could synthesize the high molecular weight of PDLLA product of 577 Da (n=8). This result indicated that the molecular weight of PDLLA product from re-polymerization process with thermo-solvent tolerant lipase from the strain A3301 was higher than PDLLA product from the controlled condition around 2.27 times. Therefore, this experiment indicated that the thermo-solvent tolerant lipase from *Streptomyces* sp. strain A3301 was able to use as bio-catalyst for PDLLA synthesis.

CHAPTER 5

DISCUSSION AND CONCLUSION

Nowadays, plastic wastes is a serious social problem because plastics barely decompose in the environment. Therefore, the biodegradable polymers are becoming more attractive so many researchers are trying to find replacements for petrochemical plastic materials. This study was focused on our group's previous actinomycete which is *Actinomadura keratinilytica* strain T16-1. These strain has high ability to produce PDLLA -degrading enzyme with enzyme activity 257 U/mL after incubation for 3 days in a 3-L airlift fermenter ([12]). The enzyme production by these strain and its scaling up have not been studied so far. Hence, this study was achieved by the scaling up of the PDLLA -degrading enzyme production through immobilized the strain T16-1 in 5-L and 10-L stirrer fermenter. Firstly, the effect of various immobilizers on the enzyme production was investigated by using scrub pad, luffa disc, sponge and calcium alginate as immobilizer. The results showed the enzyme activity of the free cells, scrub pad, luffa disc, sponge, and calcium alginate at 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 days of cultivation in 250-mL Erlenmeyer flask . 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, the use of scrub pad immobilization improved the 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 ([17, 118, 119]). 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

hours, the bacterial cells grew on the surface and inside the pores of the scrub pad. 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 ([120]). After that, the PDLLA- degrading enzyme production was scaled up to 5-L stirrer bioreactor under batch fermentation by immobilized *A. keratinilytica* strain T16-1, the effect of agitation speed on the enzyme production was studied. 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. These results indicated that increase of the agitation speed could decrease the enzyme production because of the shear force of the disc turbine on mycelium growth. 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 was determined. The maximum of enzyme activity was 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 ([121]). 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 ([122]). Stress conditions might negatively contribute to cell growth and enzyme stability ([123]). 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. These results demonstrated that the enzyme production was higher than the previously reported by Sukkhum et al. ([12]) by approximately 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 ([15, 17, 19]). 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 ([124, 125]). 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 of 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 ([124]). 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 hours of fermentation, respectively ([126]). The scale up of PDLLA-degrading enzyme production in 10-L stirrer fermenter. The result showed the productivity and yield for the enzyme production of 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 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.

The PDLLA -degrading enzyme was produced by immobilized the strain T16-1 with the highest enzyme activity of 942.67 U/mL under repeated batch fermentation from the result as showed above. This enzyme could be PLA degradation in a flask scale. The previous report showed the 6,700 mg/L PLA powder was degraded by the crude enzyme produced from the strain T16-1 under the optimized conditions: an initial enzyme activity of 200 U/mL at 60°C for 24 hours. That released 6,843 mg/L lactic acid

with 82% conversion ([22]). Thus, the scaling up of PDLLA degradation in a 5-L bioreactor by the enzyme from *A. keratinilytica* strain T16-1 under batch fermentation was investigated. The effect of agitation speeds on PDLLA degradation was determined at agitation speeds 50 and 100 rpm demonstrated lactic acid concentrations of 4,627 and 2,679 mg/L, respectively, at 60ºC after incubation for 60 hours. The percentage of conversion efficiency at agitation speeds of 50 and 100 rpm was 25 and 14, respectively. These results concluded that the increase of 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 ([122]). 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 ([69]). After that, the effect of pH on PDLLA degradation was studied. The result showed that PDLLA degradation at different pH (8.0, 9.0 and 10.0) released the lactic acid concentration of 16,651, 9,092 and 7,252 mg/L, respectively, and the percentage conversion were 89, 39 and 48, respectively, at a 50 rpm agitation rate and 60ºC. The highest PDLLA conversion efficiency was obtained at pH 8.0 after a 72 hours reaction time. The lactic concentration at pH 8.0 was higher by approximately 2.3 and 1.8 times at pH 9.0 and 10.0, respectively. This result indicated that the PDLLA degradation in 5-L bioreactor at pH 8.0, 50 rpm for 72 hours released the lactic acid concentration of 16,651 mg/L and percentage conversion was 89, which were higher than the PLA degradation in flask scale (6,700 mg/L) of about 2.5 times ([22]). The optimum pH of purified PLA degrading enzyme produced by the strain T16-1 was previously reported as 10.0 and pH stability at pH 11-12 ([10]). However, the stability of the enzyme might decreased after an extended degradation time of PDLLA at extreme conditions such as high pH or temperature. In addition, the optimum pH of the three novel PDLLA -degrading enzymes from *Amycolatopsis orientalis*, named PLAase I, II and III, were 9.5, 10.5 and 9.5, respectively ([127]). The optimum pH of the PDLLA degrading enzyme produced by *Laceyella sacchari* strain LP175 was 9.0 at 60°C ([6]).

Subsequently, the effect of lactic acid on the PDLLA degradation was studied at different concentrations (0-8,000 mg/L). The initial pH after adding lactic acid at different concentrations (0-8,000 mg/L) were 9.0, 7.43, 6.85, 4.12 and 3.80, respectively. The result showed the percentage conversion efficiency were 60, 28, 14, 6 and 5 at an initial lactic acid concentration of 0, 2000, 4000, 6000 and 8000 mg/L, respectively. This result showed the degradation product decreased when there is an increase in the initial lactic acid concentration. This phenomenon indicated that lactic acid acted as an inhibitor of the enzyme and affected the PDLLA degradation process. Owing to lactic acid being the product of an enzyme reaction which binds to the enzyme and inhibits its activity ([128]). Moreover, the initial pH of PDLLA degradation process at different lactic acid concentrations showed very low and could affected the enzyme activity. While, the optimum pH for PDLLA degradation by the enzyme produced by *A. keratinilytica* was reported as alkaline condition ([121]). Therefore, to enhance the degradation efficiency, the product inhibition effect should be considered. The method for elimination of lactic acid during PDLLA degradation was studied by using three method: A.) without dialysis, B.) separating PDLLA-degradation and dialysis and C.) Simultaneous PDLLA-degradation and dialysis. The result showed the percentage of the conversion efficiency of 62, 66 and 99 and the percentage of PDLLA powder weight loss were 14.75, 25.95 and 99.93% with experiments A, B and C, respectively. The highest % conversion efficiency and weight loss were obtained after incubation in the reaction mixture for 24 and 48 hours, respectively, and by using method C. Therefore, the most effective method for lactic acid elimination was method C with simultaneous PDLLA degradation and the dialysis method. The lactic concentration of experiment C with 18,018 mg/L was higher than those in experiments A (11,685 mg/L) and B (12,318 mg/L) by approximately 1.54- and 1.46-folds, respectively. It could be summarized that the simultaneous PDLLA degradation and dialysis method had the potential to improve the PDLLA degradation by reducing lactic acid (byproduct) at the same time as the degradation reaction. Due to the membrane pore size of the dialysis bag is 10 kDa, which is smaller than the Mw of the PDLLA-degrading enzyme (30 kDa), which can be inferred that the enzyme trapped

the enzyme inside the dialysis bag ([10]). Meanwhile, the lactic acid was diffused out of dialysis bag because it was smaller than the membrane pore size. Various previous reports demonstrated the advantage of the dialysis bag. The dialysis method was used for the recycling of homogenous AU-DENs (Au_{100}-dendrimer-encapsulated nanoparticles) catalysts in the reduction of 4-nitrophenol ([128]). 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 ([129]). However, this is the first report of enhancing PDLLA-degradation using the simultaneous PDLLA degradation and dialysis method. Commercial PDLLA powder degradation was evaluated by a crude PDLLA-degrading enzyme produced by *A. keratinilytica* strain T16-1. The percentage of weight loss of PDLLA powder degradation by the crude enzyme were 99.3%. The results revealed that the PDLLA powder had higher percent weight loss when it was degraded by using a simultaneous degradation and dialysis method with 99.3%. Meanwhile, the control experiment showed only 5% weight loss. Afterwards, other commercial PLA products such as tray, textile, spoon, glass, glass lid and straw degradation were investigated by the crude enzyme from strain T16-1 with simultaneous degradation and dialysis method. The percentage of weight loss were 32, 85.36, 6.88, 16.60, 31.45 and 3.64, respectively. This results indicated that the percentage weight loss of PLA products were lower than the PDLLA powder because the PLA product made from impurities PLA, which containing binding agents for PLA connection forming. Thus, this present work showed a potential method for PLA degradation that can be applied for reduce the plastic waste problem in the future.

However, the PDLLA degradation produced the lactic acid as product. This product was used as monomer for PDLLA re-polymerization by using bioprocess in subsequently experiment. Nevertheless, in bioprocess of PDLLA re-polymerization required the thermo-solvent tolerant lipase to be used as bio-catalyst. Hence, this experiment investigated the isolation of lipase producing actinomycetes, optimization of thermo-solvent tolerant lipase production, purification and characterization of thermo-solvent tolerant lipase. The lipase producing actinomycetes were isolated by plating

method on tween 80 agar. One hundred and thirty-one isolates were isolated from 77 soil samples at Sakerat Biospere Researves, Thailand. Among these strain, 63 isolates were identified as actinomycete based on morphological characteristics. The 23 isolates of actinomycetes had the precipitation zone on tween 80 agar of colony diameter more than 5.0 mm. The precipitation zone was indicated the lipolytic activity, a visible precipitation is the deposition of crystals of the calcium salt formed by the fatty acid liberated by the enzyme, or as a clearing of such a precipitate around colony due to complete degradation of the salt of the fatty acid ([130]). The total of 23 isolates of actinomycetes were investigated for lipase production by execution in production broth. Only 11 isolates had ability for lipase production with high activity between 60-108 U/mL. Subsequently, all 11 isolates were investigated for screening of thermo-solvent tolerant lipase producing actinomycetes. The best isolate for thermo-solvent tolerant lipase production was strain A3301, which produced the enzyme activity of 108 U/mL in production medium. The strain A3301 produced lipase of 60 and 80 U/mL (relative activity of 55% and 74%) in production medium containing 10% toluene and hexane:chloroform, respectively. Subsequently, strain A3301 has the highest lipase activity of 90 U/mL at temperature 45 -55°C and decreased the activity after increasing temperature to 65°C of 80 U/mL. According to the results, the strain A3301 was classified as thermo-solvent tolerant actinomycetes which may had the ability to produce thermo-solvent tolerant lipase. Therefore, the lipase produced from strain A3301 showed solvent tolerant and thermo-stability properties at 10-20% of toluene and hexane:chloroform with 65% and 83% of relative activity, respectively. Moreover, lipase produced by strain A3301 showed thermostability after incubated at 45°C and 55°C for 1 hours with 180 U/mL and 160 U/mL, respectively. The results showed this enzyme was stimulated at high temperature, that the enzyme activity was encouraged after incubating the enzyme at high temperature. In conclusion, these enzyme was thermo-solvent tolerant lipase and selected for optimization of the enzyme production in subsequent experiment. The first experiment for lipase production by strain A3301 to improve medium for higher lipase activity. Four medium arrangements (medium A, B, C

and D) were investigated for lipase production. The result showed the lipase activity in different production medium, the medium A, B, C (10 g/L of oil seed cake) and D (30g/L of oil seed cake) was 260, 283, 323 and 331 U/mL, respectively. The best production medium for lipase production was the medium C and D, which containing 10 and 30 g/L of oil seed cake, respectively and followed by the medium B containing rice bran oil as inducer. The lipase production in medium with inducer was higher than the medium without inducer approximately 1.2 times. This result indicated that the inducer was important to promote the enzyme production as same as the report of lipase production from *Bacillus* sp. in the presence of 1% olive oil with the maximum activity 6.0 U/mL ([115]). In addition, the lipase production by *Burkholderia multivorans* AU2 gave the highest lipase activity of 4.89 U/mL when using palm oil as inducer. Moreover, rice bran oil was also a good inducer for lipase production in this report, the total activity was not significantly different with producing by palm oil (4.38 U/mL) ([131]). However, the medium containing oil seed cake and rice bran oil were not suitable for enzyme purification in the further study because it has the small particles that make the column clogging in lipase purification step. Therefore, the medium A (without oil seed cake and rice bran oil) was selected for subsequent experiments. Though, the medium A did not have an inducer but it could be used for lipase production with 260 U/mL. Because it had other composition assisting to improve the lipase production. Lipidic carbon sources seem to be generally essential for obtaining a high lipase yield; however, a few authors have produced good yields in the absence of fats and oils([132]). Similar to lipase production from *Rhizopus niveus* IFO 4759 was investigated by using fermentation medium containing 5% peptone, 2% glucose, 0.02% yeast extract, 0.1% KH_2PO_4 and 0.05% $MgSO_4$ at pH 6.5 without any inducer ([133]). Moreover, the lipase produced from *Streptomyces* sp. CS326 was produced in culture medium containing 10 g glucose, 10 g soybean, 0.1 g Na_2HPO_4 at final volume 1L ([117]).

Various effects on lipase production were optimized such as time course, carbon sources, concentration of carbon, nitrogen sources, concentration of nitrogen, pH and temperature. The time course of lipase production by strain A3301 was

determined at different incubation times of lipase production by cultivating strain A3301 for 7 days. The maximum of lipase production was 160 U/mL after incubation at 30°C, 150 rpm for 3 days. It has been reported that the highest of lipase production (3.4 U/mL) by *Bacillus* sp. (SP5) was performed at 37°C for 48 hours ([134]). *Streptomyces* sp. produced the maximum lipase activity of 523.33 U/mL under optimized conditions at 35°C, pH 7.0 after 48 hours of incubation ([135]). Moreover, extracellular solvent tolerant lipase produced by *Pseudomonas aeruginosa* AAU2 of 0.432 U/mL after incubation at 30°C, 150 rpm for 60 hours ([108]). Production of lipase from *Burkholderia cepacia* was obtained a maximum lipase activity of 108 U/gds after 72 hours of fermentation ([136]). These results indicated that the period of lipase production was performed depends on the cell growth rate of each microorganism. Afterwards, the effect of carbon sources on lipase production were tested using dextrose, xylose, mannose, galactose, maltose and lactose. Production of lipase was the highest lipase activity of 306 U/mL in production medium containing xylose as carbon source. After that, optimization of xylose concentration on the enzyme production was performed at 0-3% (w/v) in production medium. The concentration of xylose with 1.5% (w/v) exhibited was the best concentration for lipase production by strain A3301. Lipase production was decreased when increasing concentration of xylose. This result indicated that the lipase production is significantly induced by carbon sources such as sugars, oils and other nitrogen sources ([115]). The lipase production from *Pseudomonas aeruginosa* by using medium containing dextrose as carbon source and olive oil as inducer for lipase production with the maximum activity of 41.6 U/mL. According to this report, dextrose was selected as the main component of the fermentation medium and olive oil was the best inducer whereas, dextrose was the best carbon source for lipase production. In fact, dextrose has been considered as a growth medium for microorganisms providing a satisfactory supply of nutrients for growth ([137]). It had been reported that 3% sucrose showed higher lipase production as a carbon source from *Bacillus* sp. SP5 ([134]). Moreover, *Streptomyces* sp. produced organic solvent-tolerant alkaline lipase in an optimized medium containing lactose as a carbon source ([105]). Furthermore, lipase production of

Streptomyces sp. CS326 was performed in culture medium using glucose as carbon source ([117]). Hence, the effect of nitrogen sources was investigated such as malt extract, tryptone, peptone, soy peptone, yeast extract, $NaNO_3$, $(NH_4)_2SO_4$ and KNO_3 in production medium. The strain A3301 produced lipase with the maximum lipase activity of 240 U/mL in production medium containing yeast extract as nitrogen sources. After that, the effect of yeast extract concentration on lipase production was studied at the concentration of 0-3% in production medium. 2% of yeast extract was the optimized concentration of nitrogen source for lipase production of 255 U/mL of lipase activity. In previous report, yeast extract was found to be the most suitable nitrogen source for lipase production from *Bacillus* sp. LBN4 with enzyme activity of 3.4 U/mL ([115]). Moreover, yeast extract was the best organic nitrogen source for lipase production by *Burkholderia multivorans* AU2 with the total activity of 4.70 U/mL. The organic nitrogen source plays an important role on both bacterial growth and lipase production ([131]). It had been reported that *Bacillus* sp. SP5 produced the maximum lipase activity in the medium containing 5% yeast extract as a nitrogen source ([134]). Besides, *Bacillus subtilis* PCSIRNL-39 showed maximum production of lipase by using peptone as nitrogen source ([138]). Moreover, organic solvent-tolerant alkaline lipase was produced from *Streptomyces* sp. by using an optimized medium containing soybean as a nitrogen source ([105]). Whereas, inorganic nitrogen source were not suitable for lipase production by strain A3301. Similarly, peptone, yeast extract and tryptone have been reported as suitable for high extracellular lipase production by *Pseudomonas* sp., on the other hand, inorganic nitrogen compounds cannot trigger lipase synthesis ([139]). In general microorganism utilize the organic nitrogen sources such as peptone and yeast extract for lipase production with high yields of lipase ([140]). In addition, organic and inorganic nitrogen sources played an important role in enzymes synthesis. Inorganic sources could be used quickly, while organic nitrogen sources could supply cells with growth factors and amino acid required for cell metabolism and enzyme synthesis ([141]). The effect of concentration of yeast extract on lipase production was determined. The result showed the optimum concentration of yeast extract was 2% yeast extract while

increasing concentration of nitrogen reduced the lipase production. These result indicated that the increase in concentration of nitrogen source produced higher biomass, but reduced the lipase activity ([134]). Subsequently, physicochemical factors on the enzyme production (temperature and initial pH) were studied. The effect of initial pH of production medium was investigated by adjusting production medium at different initial pH levels between pH 5.0-10.0. The maximum of lipase production of 239 U/mL at the initial pH of production medium was 7.0. Identically, the lipase production from *Burkholderia multivorans* AU2 was obtained at pH 7.0 of 4.07 U/mL. The maximum lipase activity produced from *Bacillus* sp. KS4 at pH 7.0 of 2.17 U/mL ([142]). *Acinetobacter* sp. AU07 produced an organic solvent tolerant and thermostable lipase at pH 7.0 with the maximum activity of 14.5 U/mL ([111]). These results confirmed that bacteria prefer pH around 7.0 for cell growth and lipase production ([143]). *Streptomyces* sp. produced the maximum lipase activity of 523.33 U/mL under optimized conditions at initial pH of the medium was 7.0 after 48 hours of incubation ([135]). In more detail, Stockar et al. (2006) explained that stability of enzyme catalyzed reactions is reached at specified intracellular pH values. Therefore, the changes of extracellular pH might change intracellular pH of bacterial cells that effect on destabilize enzyme synthetic network as a result, this will change cell growth and enzyme production ([131]). The last factor is temperature, the lipase production was demonstrated at various temperature between 25-40°C. The maximum of lipase production was 321 U/mL when incubated at 30°C. Similarly, production of lipase from *Bacillus* sp. KS4 was investigated at optimized temperature at 30°C with the highest lipase activity of 2.17 U/mL ([142]). *Acinetobacter* sp. AU07 produced an organic solvent tolerant and thermostable lipase at 30°C and pH 7.0 with highest activity of 14.5 U/mL ([111]). Moreover, Lipase production by *Bacillus subtilis* showed the maximum of lipase activity of 4.72 U/mL after cultivation at 30°C for 84 hours ([144]). Besides, extracellular solvent tolerant lipase produced by *Pseudomonas aeruginosa* AAU2 of 0.432 U/mL after incubation at the optimized temperature at 30°C ([108]). Temperature affected to lipase production since the cultivation at either lower or higher than the optimum temperature may lead to the decrease of bacterial growth and

enzyme production. The high temperature can cause inactivation of the enzyme metabolic pathway, while low temperature may not permit flow of nutrients across cell membrane ([131]). The properties of thermo-solvent tolerant lipase produced by strain A3301 was determined by purification and characterization. The purification of thermo-solvent tolerant lipase produced by strain A3301 was purified by loading the crude enzyme into hydrophobic chromatography and anion exchange chromatography. A summary of purification of lipase from strain A3301, the lipase was purified with a specific activity of 27,000 U/mg-protein and this purification procedure resulted in 544 fold purification of lipase. The molecular weight of purified enzyme was 45 kDa on SDS-PAGE. In previous study, Lipase from *Streptomyces* sp. (Loyola Lipase 1), *Geobacillus* sp. EPT9, *Spirulina platensis* and *Janibacter* sp. R02 had the molecular mass of purified enzyme about 45 kDa as same as the purified enzyme from strain A3301([100]). The properties of purified lipase were the optimum temperature at 60°C, thermo-stability at 30-55°C. The optimum temperature and thermo-stability of lipase produced by the strain similar to the lipase from *Geobacillus thermodenitrificans* strain AV-5 showed the optimum temperature and pH at 65°C and thermo-stability at 70°C ([145]). Moreover, the lipase produced by *Chromohalobacter* sp. LY7-8 was investigated with the optimum temperature at 60°C ([146]). Furthermore, optimum pH and pH stability of lipase produced by strain A3301 were demonstrated. The highest lipase activity was performed at pH 9.0 and could stable at pH range from 8.0-9.0. Similarly, the lipase from *Geobacillus thermodenitrificans* strain AV-5 was performed the optimum pH at pH 9.0 and pH stability at pH 10.0 ([145]). The optimum pH of lipase produced from *Chromohalobacter* sp. LY7-8 showed at pH 9.0 ([146]). The effect of organic solvents on lipase activity was determined by incubating the enzyme with several organic solvents such as acetone, chloroform, toluene, 1-butanol, isopropanol, methanol, ethanol, acetonitrite, ethyl acetate and DMSO. The lipase activity was inhibited by 1-butanol of crude A3301 lipase and purified lipase was inhibited by 1-butanol and ethyl acetate. Similarly, lipase from *Pseudomonas stutzeri* ZS04 was inhibited by n-butanol, which may be caused by structural disorders ([147]). Generally, lipases are stable in organic solvent, with some

exceptions stimulation or inhibition. Non-polar solvents shift the equilibrium from close to open form, increasing the activity of lipase. On the other hand, polar organic solvents have more harmful effects on lipase, since this type of solvent can remove some essential water layers of enzyme, changing the native conformation of lipase, through disturbing hydrogen bonding and hydrophobic interactions ([139]). The effect of 1 mM of metal ions, the lipase from strain A3301 was resistance to the presence of Li^{+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, Co^{2+}, Fe^{2+}, Cu^{2+}, Ni^{2+} and Ba^{2+}. Likewise, lipase from *Pseudomonas* sp. had resistance of $Na^{2+,}$ Ca^{2+}, Li^{+} or Sr^{2+} ([139]). The effect of 1mM of inhibitors and 0.05% of surfactant, both of crude and purified A3301 lipase was inhibited by tween85. While, the A3301 activity increased when adding Triton X-100. Lipase from *Pseudomonas aeruginosa* appeared to be strongly inhibited by adding Tween 80 and Triton WR 1339 and Triton X-100 was able to increase the lipase activity ([139]). The performance of lipases in the presence of detergents depend on some factors, such as ionic strength, pH and composition of the detergent. Triton X-100, Tween 20, and Tween 80 are nonionic polyoxyethylene detergents since they have a hydrophobic and hydrophilic part. The hydrophobic part usually consists of an alkyl chain (branched or unbranched), while the hydrophilic part is composed of uncharged ethylene oxide units. Generally, lipases have more positive effects in presence of nonionic surfactants than in presence of anionic or cationic surfactants, which can be explained by the type of binding between surfactant and lipase. The nonionic surfactants interact with lipase through hydrophobic interactions, while ionic surfactants can bind to lipases by a combination of electrostatic and hydrophobic interactions. Therefore, these combinations of interactions can promote conformational changes on the structure of lipase, decreasing the enzymatic activity ([139]). After that, the thermo-solvent tolerant lipase produced from *Streptomyces* sp. strain A3301 was applied for PDLLA polymerization. The PDLLA polymerization was performed under condensation system. The commercial lactic acid was mixed with toluene at a final concentration of 450 mg/ml, heated to 60°C, and then 10% (w/v of lactic acid) of the dried enzyme produced by strain A3301 was added into the solution at 10% (w/v of lactic acid) . The two phases reaction mixture was

continuously stirred at 60°C under a nitrogen atmosphere for 6 hours. The PDLLA polymerization conditions were optimized the effect of reaction times (8, 16 and 24 hours), temperatures (50-70°C) and concentration of dried lipase (10-30% (w/v of lactic acid)) . The best condition for PDLLA synthesis was achieved at 60°C, dried lipase concentration 10% (w/v of lactic acid) for 8 hours of reaction time and fixed lactic concentration at 450g/L under nitrogen atmosphere with the highest molecular weight of PDLLA product of 525 Da (n=7) . This result indicated that the molecular weight of PDLLA product from PDLLA polymerization with lipase was higher than the control condition about 1.1 times. This study was completed in shorter reaction time than the previous report, Lassalle and Ferreira reported the optimal conditions for biological polymerization at temperature 65°C for 96 hours by lipase under hexane condition ([148]). Chuensangjun et al (2012), reported the Mn in the range 2600-4500 of PLA was drawn from the commercial lactic acid by the commercial lipase, Lipozyme TL IM at optimum condition temperature 50°C for 5 hours in the toluene solvent and under a nitrogen atmosphere ([21]). Subsequently, the optimal condition from PDLLA polymerization experiment was applied to re-polymerization. Re-polymerization of PDLLA was investigated by using PDLLA degraded products (lactic acid) from section 2.4 as substrate. The PDLLA degraded product were dissolved in toluene at a concentration and heated to 60°C, and then 10% (w/v) of the freeze dried enzyme was added to the solution. The reaction mixture was continuously stirred at optimum condition from the experiment 4.2 under a nitrogen atmosphere. The result showed the re-polymerization process could synthesized the high molecular weight of PDLLA product of 577 Da (n=8) . This result indicated that the molecular weight of PDLLA product from re-polymerization process with thermo-solvent tolerant lipase from strain A3301 was higher than PDLLA product from the control condition around 2.27 times. This study indicated the molecular weight of PDLLA product of 577 Da (n=8) was higher than the PDLLA product from previous report of 378 Da (n=5) about 1.53 times ([22]). Therefore, this experiment indicated that this enzyme had ability to solvent tolerant at high concentration. This enzyme was able to use for PDLLA polymerization from pure lactic

acid (commercial lactic acid) and impure lactic acid produced from PDLLA degradation, that showed a molecular weight of PLA product is not significant difference. Moreover, the thermo-solvent tolerant lipase from *Streptomyces* sp. strain A3301 was able to use as bio-catalyst for PDLLA synthesis and the short oligomer could be applied as substrate for PDLLA synthesis in the other experiments.

In conclusion, this thesis mainly focused on completing the biological recycling process for PDLLA using the enzyme produced by actinomycete. In the first section, the PDLLA-degrading enzyme production by strain T16-1 was successfully scaled-up to 5-L and 10-L stirrer fermenters. The results showed that a scrub pad was 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 hours of cultivation time. Batch, repeated-batch, and continuous fermentation processes were 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 was demonstrated with an enzyme activity of 578.67 U/mL and productivity of 12.06 U/mL·h. Subsequently, The PDLLA-degrading enzyme produced from *Actinomadura keratinilytica* strain T16-1 was applied for PDLLA degradation in a 5-L stirrer bioreactor under a batch condition. The result indicated that the best condition for PDLLA -degradation was an agitation speed of 50 rpm at 60ºC under a control pH 8.0. The maximum percentage of the conversion efficiency was 89 after incubation for 72 hours. Moreover, the effect of lactic acid as a product inhibitor was examined. The percentage of PDLLA conversion efficiency is decreased when increasing the initial lactic acid concentration. Thus, the best method to remove lactic acid during PDLLA degradation should be demonstrated. As a result, the simultaneous PDLLA degradation and dialysis method showed the highest percent weight loss in both reactions containing PDLLA powder and commercial PDLLA materials. In the second part, we focus on re-polymerization process by using thermo-solvent tolerant lipase produced by

isolated actinomycetes. Lipase producing actinomycetes were isolated from soil sample of Sakerat Environmental Research Station Sakerat Biophere Reserves, Thailand. The best lipase production was isolate A3301 of 321 U/mL in optimum production medium containing xylose as carbon source at concentration of 1.5% (w/v), yeast extract as nitrogen source at concentration of 2% (w/v), initial pH as 7.0, incubated at 30°C, 150 rpm for 3 days. The strain A3301 has ability for solvent tolerant at 10-20% of toluene or hexane:chloroform with relative activity of 65% and 83%, respectively Moreover, it showed thermostability at 45-65°C with relative activity of 100%, and 88%, respectively. The characteristic of the isolate A3301 were shown the colony appeared to be circular and has yellow spore on ISP-2 agar. Isolate A3301 was a Gram-positive bacteria, LL-diaminopimelic acid of the cell wall type. Color of aerial and substrate mycelium on ISP1-ISP5 were grey-yellow, yellow-yellow, grey-white, white-white and white-yellow, respectively. Spore surfaces were smooth plate. It can utilized lipid, skim-milk and nitrate but cannot use cellulose and gelatin. It indicated that isolate A3301 can produce lipase and protease but could not produced cellulase and gelatinase. Moreover, it had ability to NaCl tolerate at concentration of 0-7% in ISP2 agar. Isolate A3301 can use various carbon source such as dextrose, xylose, mannose, galactose, maltose and lactose. Moreover, the strain A3301 was identified as *Streptomyces* sp. with a high levels of 16s rRNA gene sequence. The lipase from *Streptomyces* sp. strain A3301 was purified by hydrophobic chromatography and anion exchange chromatography. The purified A3301 lipase with a specific activity of 27,000 U/mg-protein and a purification fold of 544 folds were obtained. A single band with an obvious molecular mass of ~45kDa on SDS-PAGE with CBB staining. The properties of the purified lipase were the optimum temperature was 60°C and the enzyme exhibited at a wide range of thermostable at temperature 30°C-55°C. The optimum pH of lipase from strain A3301 was pH 9.0 and the pH stability at pH 8.0-9.0. The lipase activity was inhibited by 1-butanol and ethyl acetate, the effect of 1mM of metal ions, 1mM of inhibitors and 0.05% of surfactant that was inhibited by tween 85. After that, thermo-solvent tolerant lipase was applied for PDLLA polymerization. The optimal condition for PDLLA synthesis

was achieved at 60°C, dried lipase concentration 10% (w/v of lactic acid) for 8 hours of reaction time and fixed lactic concentration at 450g/L under nitrogen atmosphere with the highest molecular weight of PDLLA product of 525 Da (n=7). Subsequently, the re-polymerization process could synthesized the high molecular weight of PDLLA product of 577 Da (n=8) under optimum condition ([22]).

Therefore, this study showed the PDLLA re-polymerization by biological process including PDLLA degradation and PDLLA polymerization. Protease from *Actinomadular keratinilytica* strain T16-1 was investigated to convert PDLLA to lactic acid monomer in PDLLA degradation process. After that, PDLLA degraded product (lactic acid) was used as substrate for PDLLA polymerization that was performed by using lipase produced by *Streptomyces* sp. strain A3301 as catalyst. This thesis could use as a model to complete the biological recycling of PDLLA.

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APPENDIX

-Appendix A1 ISP2 medium (Yeast extract-malt extract agar)

Yeast extract 4 g

Malt extract 10 g

Dextrose 4 g

Distilled water 1 L

Agar 15 g

Adjust to pH 7.3

Liquefy agar by steaming at 121 ºC for 15-20 minutes. Dispense appropriate amount for a slanting into at least 6 tubes for each culture. Sterilize by autoclaving; cool tubes as slants. Use the agar for preparation of stock cultures. Also sterilize medium 2 in flasks for pouring the sterilized medium into Petri dishes.

-Appendix A2 Fermentation medium for PLA-degrading enzyme production (Basal medium)

$(NH_4)_2SO_4$ 4 g

K_2HPO_4 4 g

KH_2PO_4 2 g

$MgSO_4.7H_2O$ 0.2 g

Gelatin 2.38 g

PLA powder 0.35 g

Distilled water 1 L

Sterilized by steaming at 121ºC for 15-20minutes. Dispense appropriate amount into 50 mL per flasks at least 3 flasks for each culture.

-Appendix A3 Enrichment medium for lipase production

Peptone	0.05 g
KH_2PO_4	0.15 g
Na_2HPO_4	0.1 g
$MgSO_4$	0.05 g
$(NH_4)_2SO_4$	0.5 g
NaCl	0.05 g
Tween 80	12 mL
Distilled water	100 mL

Liquefy agar by steaming at 121ºC for 15-20min. Dispense appropriate amount into 50 mL per flasks at least 3 flasks for each culture.

-Appendix A4 Tween 80 agar

Solution I:

Peptone	10 g
NaCl	5 g
$CaCl.H_2O$	0.1 g
Agar	15 g
Distilled water	900 mL

Solution II:

Tween 80	10 mL
Distilled water	90 mL

Mix solution I and solution II after sterilized by steaming at 121ºC for 15-20 minutes and pouring into petri dishes.

-Appendix A5 Starch casein agar

Soluble starch 10 g

K_2HPO_4 2 g

KNO_3 2 g

Casein 0.3 g

$MgSO_4.7H_2O$ 0.05 g

$CaCO_3$ 0.02 g

$FeSO_4.7H_2O$ 0.01 g

Distilled water 1 L

Agar 15 g

Adjust to pH 7.0

Liquefy agar by steaming at 121ºC for 15-20minutes. Sterilize in flasks for pouring into Petri dishes.

-Appendix A6 Production medium for lipase

Corn oil 0.5 mL

NaCl 0.16 g

$(NH_4)_2SO_4$ 0.12 g

Distilled water 100 mL

Adjust to pH 7.0

Sterilized by steaming at 121ºC for 15-20minutes. Dispense appropriate amount into 50 mL per flasks at least 3 flasks for each culture.

-Appendix A7 ISP3 medium (Oatmeal medium)

Oatmeal 20 g

Agar 18 g

Distilled water 1 L

-Trace salts solution (Use as directed in ISP-3, 4 and 5)

$FeSO_4.7H_2O$ 0.1 g

$MnCl_2.4H_2O$ 0.1 g

$ZnSO_4.7H_2O$ 0.1 g

Distilled water 100 ml

Cook or steam 20 g oatmeal in 1,000 ml distilled water for 20 minutes Filtrate, add distilled water to restore volume of filtrate to 1,000 ml, add trace salts solution 1 ml. Adjust to pH 7.2 with NaOH. Add 18 g agar; liquefy by steaming at 100ºC for 15-20 minutes. Sterilize in flasks for pouring into Petri dishes. Swirl medium before pouring to assure even distribution of the oatmeal.

-Appendix A8 ISP4 medium (Inorganic salt-starch agar)

Solution I: soluble starch 10 g

Make a paste of the starch with a small amount of cold distilled water and bring to a volume of 500 mL

Solution II:

K_2HPO_4 1 g

$MgSO_4.7H_2O$ 1 g

NaCl 1 g

$(NH_4)_2SO_4$ 2 g

$CaCO_3$ 2 g

Distilled water 500 mL

Trace salts solution 1 ml

Adjust to pH 7.0-7.4

Mix starch suspension and salts solution. Add agar 15 g. Liquefy agar by steaming at 121ºC for 15-20 minutes. Sterilize in flasks for pouring into petri dishes.

-Appendix A9 ISP5 medium (Glycerol-asparagine agar)

L-asparagine 1 g

Glycerol 10 g

K_2HPO_4 1 g

Distilled water 1 L

Trace salts solution 1 ml

Agar 15 g

Adjust to pH 7.0-7.4

Liquefy agar by steaming at 121ºC for 15-20 minutes. Sterilize in flasks for pouring into Petri dishes.

Pridham and Gottlieb (1948) trace salts: 100 ml

$CuSO_4.5H_2O$ 0.64 g

$FeSO_4.7H_2O$ 0.11 g

$MnCl_2.4H_2O$ 0.79 g

$ZnSO_4.7H_2O$ 0.15 g

Distilled water 100 mL

Only 1 ml of this solution was used per liter of final medium. Stored at 3-5°C until required for use. Bring to room temperature before use. Prepare fresh solution each month.

-Appendix A10 ISP9 medium

$(NH_4)_2SO_4$ 2.64 g

K_2HPO_4 2.38 g

KH_2PO_4 5.65 g

$MgSO_4.7H_2O$ 1 g

Pridham Gottieb trace element 1 mL

Agar 15 g

Distilled water 1 L

Adjust to pH 6.8-7.0

Liquefy agar by steaming at 121ºC for 15-20 minutes. Sterilize in flasks and mix with filtrated carbon source for pouring into Petri dishes.

-Appendix A11 CMC agar (Cellulose medium)

Cellulose 10 g

Yeast extract 2.4 g

Peptone 5.1 g

NaCl 5.1 g

$CaCl_2$ 1.5 g

Distilled water 1 L

Liquefy agar by steaming at 121ºC for 15-20min. Sterilize in flasks for pouring into Petri dishes.

-Appendix A12 Gelatin medium

Glucose 2 g

Peptone 0.5 g

Gelatin 20 g

Adjust to pH 7.0

Liquefy agar by steaming at 121ºC for 15-20min. Dispense appropriate amount for a tube into at least 3 tubes for each culture. Sterilize by autoclaving and cool tubes for gelatinase production assay.

-Appendix A13 Skim milk medium

Skim milk powder 28 g

Casein enzymic hydrolysate 5 g

Yeast extract 2.5 g

Dextrose 1 g

Agar 15 g

Adjust to pH 7.0

Liquefy agar by steaming at 110ºC for 15-20min. Sterilize in flasks for pouring into Petri dishes.

-Appendix B1 100mM Tris-HCl pH 9.0

0.2 M Tris base 1,000 mL

2 M HCl 10 mL

Distilled water 2,990 mL

Adjust pH to 9.0 with HCl

-Appendix B2 Composition of reagents of DNA extraction was as follows:

-TE buffer :

Tris-HCl 10.8 g

EDTA-2Na 0.83 g

pH was adjusted to 8.0 with HCl

Add distilled water to 1,000 ml

- TAE buffer:(10X)

Tris-base 12.1 g

100% acetic acid 2.9 ml

0.5 M EDTA (pH 8.0) 5 ml

Add distilled water to 250 ml

Lysozyme 50 mg/ml in water

Proteinase K:20 mg/ml in water 10% SDS

5M NaCl Chloroform

isopropanol

70% ethanol

-Appendix B3 protein concentration assay

Complex-forming reagent: Prepare immediately before use by mixing the following stock solutions in the proportion 100:1:1 (by volume), respectively:

Solution A: 2% (w/v) Na_2CO_3 in distilled water.

Solution B: 1% (w/v) $CuSO_4.5H_2O$ in distilled water.

Solution C: 2% (w/v) sodium potassium tartrate in distilled water.

- Folin reagent (commercially available): Used at 1N concentration.

- Standard: Use a stock solution of standard protein (e.g., bovine serum albumin fraction V) containing 2 mg/ml protein in distilled water, stored frozen at -20°C. Prepare standards by diluting the stock solution with distilled water as follows:

Stock solution (µl):	0	5	25	50	250	500
Water (µl):	500	495	475	450	250	0
Protein conc. (µg/ml):	0	20	100	200	1000	2000

One milliter freshly mixed complex-forming reagent was added to 0.1 ml of sample or standard. Let the solution stand at room temperature for 10 min. Add 0.1 ml of Folin reagent, using a vortex mixer, and let the mixture stand at room temperature for 30-40 minutes. Read the absorbance at 750 nm if the protein concentration was below 500 µg/ml. Plot a standard curve of absorbance as a function of initial protein concentration and use it to determine the unknown protein concentrations.

-Appendix B4 Composition of reagents of SDS-PAGE was as follows:

The precipitated enzyme was dissolved in sample buffer.

1. Acrylamide-bis Stock, 100 ml:

30% acrylamide

0.8% N,N'-methylene bis acrylamide

2. 2x SDS-Running Buffer, 100 ml:

0.75 M Tris-HCl, pH 8.8

0.2% SDS

3. 2x SDS-Stacking Buffer, 100 ml:

0.25 M Tris-HCl, pH 6.8

0.2% SDS

4. 5x SDS-Electrode Buffer, 100 ml:

0.125 M Tris-HCl, pH 8.3

0.96 M glycine 0.5% SDS

5. TEMED full strength

6. 2x SDS-SAB

0.125 M Tris-HCl, pH 6.8

4% SDS

20% glycerol

0.002% Bromphenol blue

10% mercaptoethanol

(add just before use)

Compositions of coomassie brilliant blue stain and destain solution were as follows:

7. Stain:

450 mL water

500 mL methanol

75 mL acetic acid

5 g Coomassie brilliant blue

8. Destain:

1.0 L water

1.0 L methanol

200 L acetic acid

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