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New Insight into Biodegradation of Poly (L-Lactide), Enzyme Production and Characterization

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1. Introduction

Owing to the global utilization of plastics in large quantities, their disposals as solid waste causes deleterious effect on the environment and global warming occurrence. The development of biodegradable plastics is considered to be a product innovation that can help to resolve the problems of plastic waste. The use of biodegradable plastic is one of method to resolve these problems. Biodegradable polymers were classified into four groups depend on the resources of monomers production such as agro-polymer (cellulose, chitin or starch), produced through fermentation by microorganisms (Polyhydroxyalkanoates, PHA), obtained by petrochemical products (Polycaprolactone, PCL) and conventional synthesis from bio-derived monomer (polylactic acid, PLA). In 2002, Cagill Dow was the global leader in commercialization of PLA production, launched a 300 million-USD effort to begin mass production of plastic based on PLA under the trade name Nature work™. The branded PLA is a compostable polymer used in a wide range of applications. Thus, PLA is expected to replace presently used plastic material synthesized from petrochemicals. Recently, the plastic compost by microbes has become a method of interest for plastic waste treatment. After worldwide use of PLA and disposal of PLA plastic waste, recycling of the PLA waste is necessary for utilizing materials efficiently. Biological processes by both microbial and enzymatic activities are currently considered to be sustainable recycling methods for PLA. Recently, several actinomycetes and thermophilic bacteria have been reported to exhibit PLA-degrading ability such as *Brevibacillus*, *Bacillus smithii*, *Geobacillus*, and *Bacillus licheniformis* (Tomita et al., 1999; Sakai et al., 2001; Tomita et al., 2004; Kim et al., 2007). Various reports on PLA-degrading enzyme were investigated such as protease, lipase or hydrolase. Proteinase K, a fungal serine protease of *Tritirachium album* has received attention since the early study by Williams (1981). At present study, a new incident on the production of enzyme by using statistical method was reported by Sukkhum et al. (2009b). The improvement of PLA-degrading enzyme production was successful and could be scale-up in 3L airlift fermenter. At this time, the available information on PLA-degrading microorganisms and enzymes are still less than that available other biodegradable plastics such as PCL or PHB. Thus, the study on PLA degradability and application of the enzyme for recycling of commercial PLA are very interesting in recent year. This article summarized

and updated new insight into PLA-degrading microorganisms, development of enzyme production and characterization as well as demonstrated the biological recycling of PLA from various kinds of bacteria.

2. Poly(L-lactide), poly(Lactic acid), PLA

Poly(L-lactide) or PLA is one of biodegradable plastics, synthesized from lactic acid which can be produced from farm and agricultural product such as cassava, rice, corn and corncob by bacterial or fungal fermentation. Lactic acid, $\text{HOCH}_2\text{CHCOOH}$, exists as two enantiomers, L- and D- lactic acid that involves in the PLA processing and polymerization. Polymerization of lactic acid to high molecular weight PLA can be achieved by two ways: direct condensation and ring opening polymerization (Vink et al., 2002). Commercial PLA are copolymers of L- and D- lactides. Usually, L- isomer is the main product of L-lactic acid from biological sources such as bacterial and fungal fermentation. At present, Cargill Dow Polymers operates the world's largest PLA production from renewable resources. This company efforts at developing a comprehensive life cycle inventory for PLA pellet production span several years. Fig. 1 is a simplified flow and system boundary diagram for PLA production. The analysis depicted include impact associate with corn growing, transport of corn to the corn wet mill, processing of corn into dextrose by enzyme hydrolysis, conversion of dextrose into lactic acid by fermentation method, conversion of lactic acid into lactide and polymerization of lactide into polylactide by condensation and ring opening polymerization (Vink et al., 2002). PLA can be used for various applications. For example PLA make the fibre suitable for technical textile application especially for apparel and has inspired several studies on controlled drug delivery systems or surgical sutures are wound closure filaments bricated in various shapes (Wood, 1980; Laitinen et al., 1992) as well as Cargill Dow's Nature Works™ branded PLA is a compostable polymer used in a wide range of packaging (primarily for food), film, bottles and fibre applications such as short shelf life milk and oil packaging or cold drink cup.

3. Microbial degradation of PLA

The degradation of PLA by actinomycetes, Pranamuda et al. (1997) reported the ability of *Amycolatopsis* strain HT-32 formed clear zone on PLA plate. After their finding, several PLA-degrading microorganisms were recorded as show in the Table 1. Moreover, 15 strain of *Amycolatopsis* sp. formed clear zones on PLA agar plate, showing a large distribution of PLA-degrading actinomycetes within this genus (Pranamuda & Tokiwa, 1999). Ikura & Kudo (1999) isolated *Amycolatopsis* sp. strain 3118 from supernatant pond water or river water demonstrated PLA-degrading activity.

In the same time, *Amycolatopsis* sp. strain KT-5-9 was isolated and showed the degradability on PLA and silk fibroin (Tokiwa et al., 1999). Nakamura et al. (2001) also isolated and identified PLA degrading strain as *Amycolatopsis* sp. strain K104-1 and K104-2. Jarerat et al. (2002) investigated the distribution of PLA-degrading actinomycetes. Among 41 genera (105 strains) of tested actinomycetes found that PLA-degrading strain were limited to the family *Pseudonocardiaceae* and related genera such as *Amycolatopsis*, *Lentzea*, *Kibdelosporangium*, *Streptoalloteichus* and *Saccharothrix* (Jarerat et al., 2002). Interestingly, Sukkhum et al. (2009a) recently reported that PLA-degrading actinomycetes were not limited to this family. The results concluded that PLA-degrading strains were distributed to various families e.g.

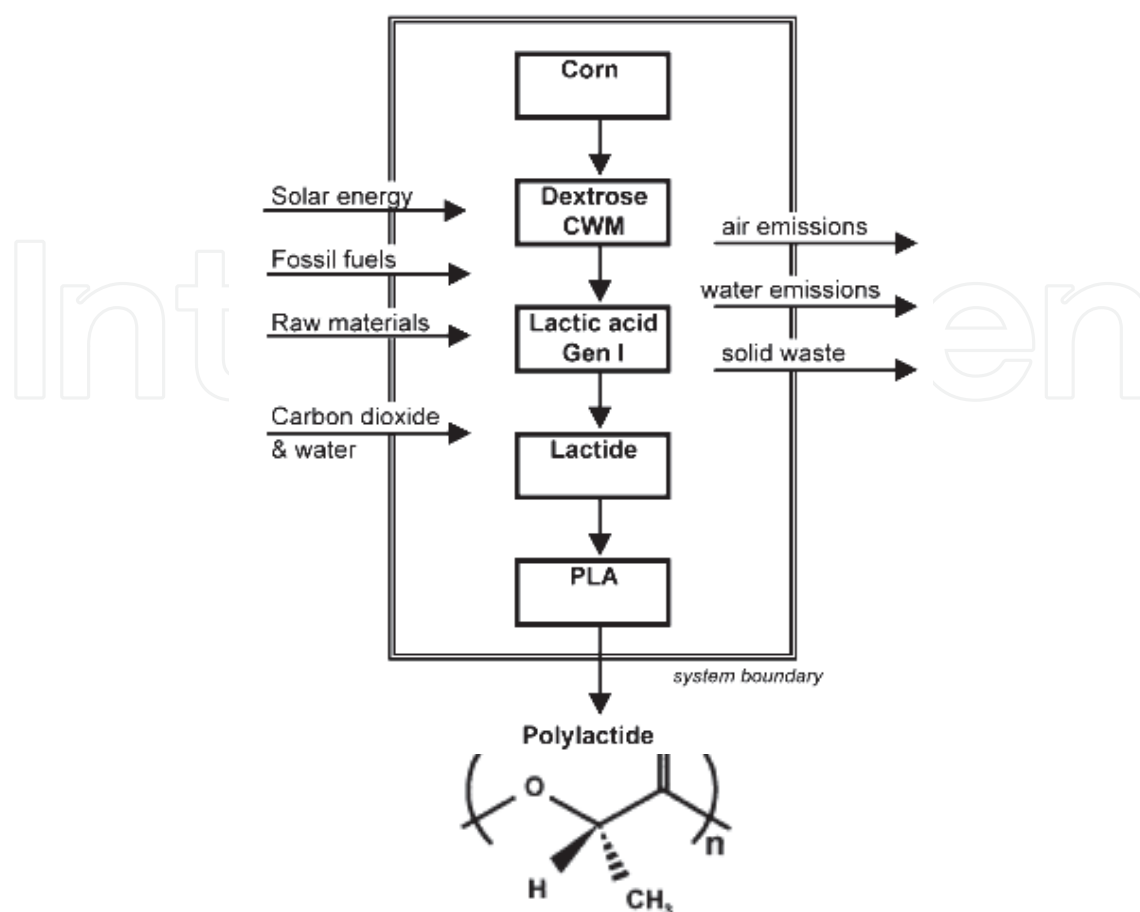


Fig. 1. PLA manufacturing overview of Cargill Dow under commercial name NatureWorks™ (Vink et al., 2002)

Thermomonosporaceae, *Micromonosporaceae*, *Streptosporangiaceae*, *Bacillaceae* and *Thermoactinomycetaceae* (Fig. 2). Among 13 isolates, strain T16-1 showed the highest PLA-degrading activity at 50°C and identified as *Actinomadura keratinilytica* strain T16-1. This finding draw a distinction to the report of Jarerat et al. (2002) that several actinomycetes limited only in the family *Pseudonocardiaceae* and related genera were capable of degrading PLA. Furthermore, strain T16-1 was further identified by Sukkhum et al. (2009a). It formed cream-yellow substrate mycelium on ISP-2, 4 and 5. Aerial mycelium rarely forms. The color of aerial mycelium was green on ISP-2 and ISP-4, and white on ISP-3. Oligosporic curved chains of spinies spores were borne on aerial hyphae. The temperature ranges for growth were 30-60 °C. The strain could degrade skim milk and tween 80. Cell hydrolysates contained galactose, glucose, madurose, mannose and ribose. Phospholipids include diphosphatidylglycerol, phosphatidylinositol, phosphatidylglycerol and phosphatidylinositol mannoside. The major cellular fatty acids were iso C_{16:0} and iso C_{17:0}. The principal menaquinones were MK-9(H₄), MK-9(H₆) and MK-9(H₈). DNA G+C content of the the strain was 72.2 mol%. Strain T16-1 showed 85% relatedness with *A. keratinilytica* WCC-2265^T. In agreement with phenotypic, chemotaxonomic and 16S rDNA sequencing, it could be concluded that the strain was identified as *Actinomadura keratinilytica*, a novel actinomycetes which produced high PLA-degrading activity. Several PLA-degrading bacteria at high temperature (≥50 °C) have been reported. A thermophilic strain, *Brevibacillus* sp. which degrades L-PLA film at 60°C was isolated from soil (Tomita et al., 1999).

Strain	Detection method of PLA degradation	Reference
<i>Amycolatopsis</i> sp. HT 32	Film-weight loss; monomer production	Pranamuda et al., 1997
<i>Amycolatopsis</i> sp. 3118	Film-weight loss; monomer production	Ikura & Kudo, 1999
<i>Amycolatopsis</i> sp. KT-s-9	Clear zone method	Tokiwa et al.,1999
<i>Amycolatopsis</i> sp. 41	Film-weight loss; monomer production	Pranamuda et al., 2001
<i>Amycolatopsis</i> sp. K104-1	Turbidity method	Nakamura et al., 2001
<i>Lentzea waywayandensis</i>	Film-weight loss; monomer production	Jarerat & Tokiwa, 2003
<i>Kibdelosporangium aridum</i>	Film-weight loss; monomer production	Jarerat et al., 2003
<i>Tritirachium album</i> ATCC 22563	Film-weight loss; monomer production	Jarerat & Tokiwa,2001
<i>Brevibacillus</i>	Change in molecular production and viscosity	Tomita et al., 1999
<i>Bacillus stearothermophilus</i>	Change in molecular production and viscosity	Tomita et al., 2003
<i>Bacillus smithii</i> PL 21	Change in molecular production and viscosity	Tomita et al., 2004
<i>Bacillus licheniformis</i> PLLA-2	Biodegradation test	Kim et al., 2007
<i>Paenibacillus amylolyticus</i> TB-13	Turbidity method	Shigeno et al., 2003
<i>Bacillus clausii</i> strain pLA-M4	Molecular technique	Mayumi et al., 2008
<i>Bacillus cereus</i> pLA-M7	Molecular technique	Mayumi et al., 2008
<i>Treponema denticola</i> pLA-M9	Molecular technique	Mayumi et al., 2008
<i>Paecilomyces</i>	Molecular technique	Sangwan & Wu, 2008
<i>Thermomonospora</i>	Molecular technique	Sangwan & Wu, 2008
<i>Thermopolyspora</i>	Molecular technique	Sangwan & Wu, 2008
<i>Actinomadura keratinilytica</i> T16-1	Clear zone and turbidity method	Sukkhum et al., 2009a
<i>Micromonospora echinospora</i> B12-1	Clear zone and turbidity method	Sukkhum et al., 2009a
<i>Micromonospora viridifaciens</i> B7-3	Clear zone and turbidity method	Sukkhum et al., 2009a

Strain	Detection method of PLA degradation	Reference
<i>Nonomuraea terrinata</i> L44-1	Clear zone and turbidity method	Sukkhum et al., 2009a
<i>Nonomuraea fastidiosa</i> T9-1	Clear zone and turbidity method	Sukkhum et al., 2009a
<i>Bacillus licheniformis</i> T6-1	Clear zone and turbidity method	Sukkhum et al., 2009a
<i>Laceyella Sacchari</i> T11-7	Clear zone and turbidity method	Sukkhum et al., 2009a
<i>Thermoactinomyces vulgaris</i> T7-1	Clear zone and turbidity method	Sukkhum et al., 2009a

Table 1. PLA-degrading microorganisms, type of enzyme and detection method for PLA degradation

Sakai et al. (2001) isolated thermophilic L-PLA-degrading bacteria from a garbage fermentor, identified as *Bacillus smithii*. The strain grew well in the medium containing 1% L-PLA and the molecular weight of L-PLA decreased by 35.6% after 3 days incubation with shaking at 60°C. Another isolation of L-PLA-degrading thermophile *Geobacillus* sp. strain 41 was reported by Tomita et al. (2004). The time course of L-PLA degradation was monitored at 60°C for 20 days and degradation was confirmed by the change in molecular weight and viscosity of the residual polymer. Newly thermophilic bacteria isolated from compost was isolated and identified as *Bacillus licheniformis*. It degraded not only low-molecular weight PLLA but also other PLLAs having higher molecular weight at 58°C (Kim et al., 2007). Recently, various molecular analyses such as 16S rDNA studies have confirmed that only less than 1% of microorganisms in the natural environment can be cultured by traditional culture-based methods (Bintrim et al., 1997; Rondon et al., 1999). Mayumi et al. (2008) suggested that some un-culturable microorganisms may also be associated with PLA degradation. Metagenomic library consisting of the DNA extraction from PLA disks buried in compost was constructed and identified three PLA-degrading genes which worked at high temperature (70°C). Afterward, screening of un-culturable microorganism’s technique was also used. Gene sequences from genera *Paecilomyces*, *Thermomonospora*, and *Thermopolyspora* which play an important role in PLA degradation were most abundant in the compost sample containing PLA (Sangwan & Wu, 2008). Almost of reported PLA-degrading microorganisms are shown in the Table 1.

4. PLA-degrading enzyme purification and characterization

Usually, the enzymes play a significant role in degradation of polymers, even though the enzymes are not only responsible for the hydrolysis of polymers. The enzymatic degradation of aliphatic polyesters by hydrolysis is a two-step process. The first step is adsorption of the enzyme on the surface of the substrate through surface-binding domain and the second step is hydrolysis of the ester bond (Tokiwa & Calabia, 2006). Williams (1981) first reported the degradation of L-PLA by proteinase K from *T. album*. Naturally occurring amino acids as constituents of protein are L-isomers. As reported by Reeve et al. (1994) proteinase K was not able to cleave D-stereoisomer of PLA. It seems reasonable to conclude that PLA-degrading enzyme is protease-type enzyme which recognizes the repeated L-lactic acid unit of PLA as L-alanine unit of silk fibroin (protein). Currently, a few

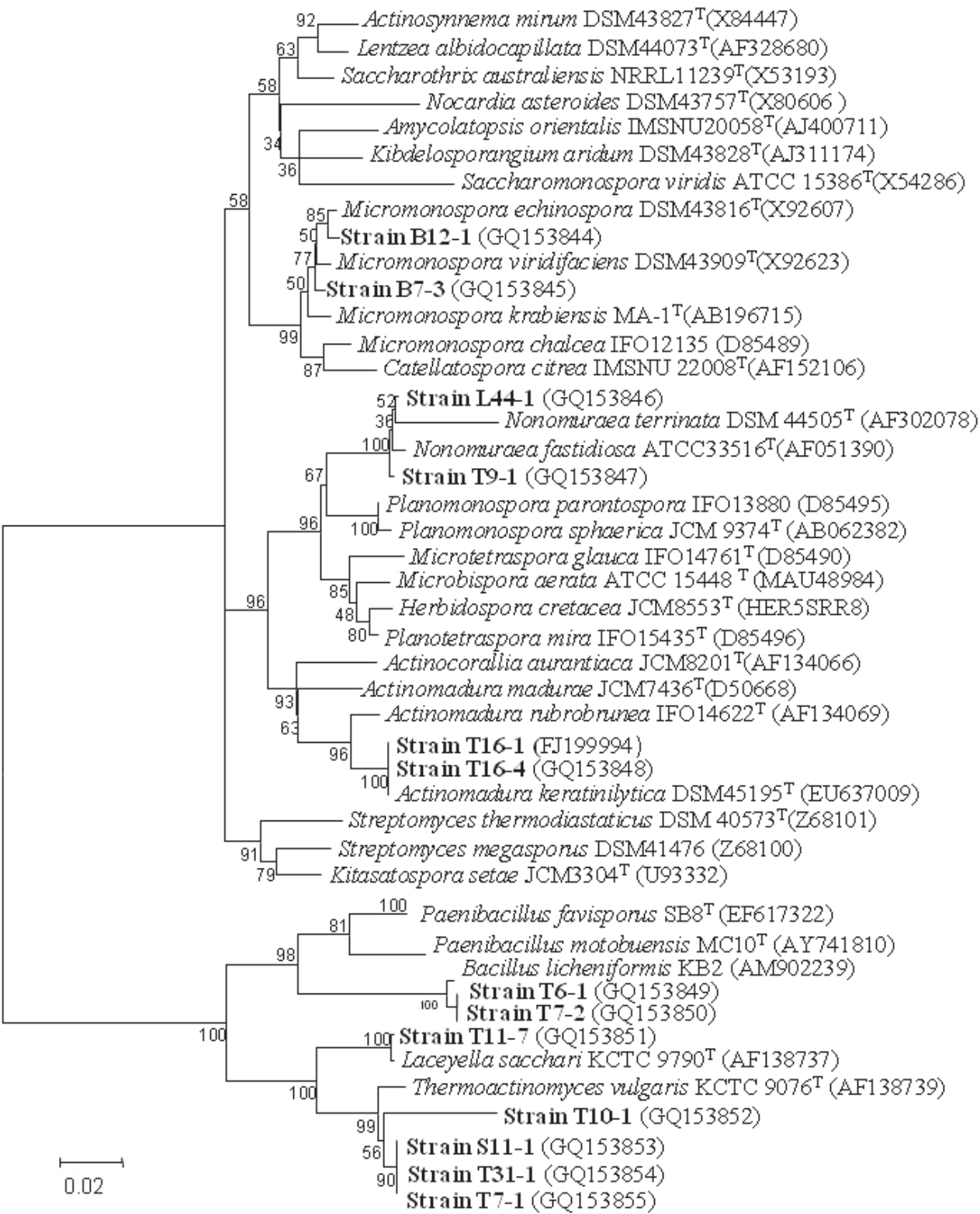


Fig. 2. Phylogenetic positions of PLA-degrading microorganisms. The phylogenetic tree was constructed by the neighbor-joining method. Bootstrap values at branching points are expressed as percentages from 1000 replications. The scale bar indicates 0.02 substitutions per nucleotide position. T = type strain. (Sukkhum et al., 2009a)

Strain	Mw (kDa)	Optimum pH and temperature	Substrate specificity	Enzyme type	Reference
<i>Amycolatopsis</i> sp. 41	40	pH 6.0 37-45°C	casein, silk powder, Suc-(Ala) ₃ -pNA	protease	Pranamuda et al., 2001
<i>Amycolatopsis</i> sp. K104-1	24	pH 9.5 55-60°C	Casein, fibroin	Serine protease	Nakamura et al., 2001
<i>T. album</i>	-	-	silk fibroin, elastin, (Suc-(Ala) ₃ -pNA)	protease	Jarerat & Tokiwa, 2001
<i>B. smithii</i>	62.5	pH 5.5 60°C	pNP-butyrate, capryrate, laurate, palmitate, stearate	acyltransferase	Sakai et al., 2001
<i>Cryptococcus</i> sp. S-2	20.9	-	L-PLA , PBS, PCL, PHB	Lipase	Masaki et al., 2005
<i>Amycolatopsis orientalis</i> ssp. <i>orientalis</i>	24, 19.5, 18	pH 9.5, pH 10.5 pH 9.5 50-60°C	PLA, casein, C8 ester	Serine protease	Li et al., 2008
<i>Actinomadura keratinilytica</i> T16-1	30	pH 10.0 70°C	PLA, (Suc-(Ala) ₃ -pNA), gelatin	Serine protease	Sukkhum et al., 2009a

Table 2. The characteristics of purified PLA-degrading enzyme from various strains

reports on purification and characterization of PLA-degrading enzyme have been available (Table 2). Pranamuda et al. (2001) reported the purification of PLA-degrading enzyme from *Amycolatopsis* strain-41, with molecular weight of 43 kDa from size exclusion chromatography or 40 kDa from SDS-PAGE analysis. Optimum pH and temperature were 6.0 and 37-45°C, respectively.

Besides PLLA, the enzyme degrades casein, silk powder and Suc-(Ala)₃-pNA at an even lower level than Proteinase-K, but not Suc-(Gly)₃-pNA, poly (ε-caprolactone) and poly(β-hydroxybutyrate).The PLA-degrading enzyme of this genus with higher substrate specificity on PLA depolymerase produced by *Amycolatopsis* strain K104-1 was reported by Nakamura et al. (2001). The enzyme was classified as a serine type protease having molecular weight of 24 kDa, which exhibited degrading activity on casein and fibroin, but not collagen-type I, PCL and PHB. The optimum pH for enzyme activity is 9.5, and the optimum temperature is 55-60°C. In addition to this, the culture supernatant of *T. album* with 0.1% gelatin also showed hydrolytic activity on silk fibroin, elastin and (Suc-(Ala)₃-pNA) but not on PCL, PHB and PBS. From the results of a substrate specificity study, it was concluded that the enzyme of *T. album* might be protease rather than lipase or PHB depolymerase (Jarerat & Tokiwa, 2001). In addition, Sakai et al. (2001) purified an enzyme

with a molecular weight of 62.5 kDa from a thermophilic *B. smithii*. This enzyme could be degraded various kinds of fatty acid esters and the molecular weight of L-PLA decreased at 60°C. However, control was not included in the experiment. A cutinase-like enzyme from the yeast *Cryptococcus* sp. strain S-2 was purified. The enzyme has a molecular weight of 20.9 kDa and could degrade L-PLA as well as other synthetic polymers such as PBS, PCL, and PHB (Masaki et al., 2005). Afterward, three novels purified PLA-degrading enzymes produced by *Amycolatopsis orientalis* ssp. *orientalis* named PLAase I, II and III, were purified. The molecular masses of these three PLAases as determined by SDS-PAGE were 24.0, 19.5 and 18.0 kDa, with the pH optima being 9.5, 10.5 and 9.5, respectively. The optimal temperature for the enzyme activities was 50–60°C (Li et al., 2008). Recently, Sukkhum et al., (2009a) reported that PLA-degrading enzyme was produced by *A. keratinilytica* strain T16-1 in liquid medium. Crude enzyme was purified by using DEAE-Toyopearl 650C and DEAE-Toyopearl 650S. The enzyme was purified to 13 folds with a recovery of 24% and a specific activity of 38.3 U/mg protein. The molecular weight of purified enzyme was approximately 30 kDa. The optimum pH and temperature were 10.0 and 70°C, respectively. The enzyme was stable at pH 11-12. Moreover, 70% of the enzyme activity remains when incubated at 70°C for 1h. The purified enzyme was inhibited by 5mM EDTA and 5mM Phenylmethyl sulfonyl fluoride as well as diisopropyl fluorophosphates, strongly hydrolyzed Suc-(Ala)₃-pNA, gelatin and PLA, but show low activity on casein. The n-terminal amino acid sequence of pure protein was determined for the initial 15 residues as follow: GYQNNPPSAGLDRAA which was different from that of other registered PLA-degrading enzymes but similar to serine protease from *Streptomyces avermitilis* (83% identity) and 81% identity with alkaline serine protease from *Streptomyces pristinaespiralis* ATCC 25486. Many PLA-degrading enzymes were identified as serine type protease. For example, n-terminal amino acid sequences of purified PLA-degrading enzyme from *Amycolatopsis orientalis* ssp. *orientalis*, PLAase III (YDVRGGDAYYINNSS) demonstrated the 86% identity with serine protease from *Streptomyces lividan* and a serine protease precursor of *Streptomyces coelicolor* A3 (Li et al., 2008). Pure PLA-degrading enzyme from *Amycolatopsis* sp. K104-1 was also classified by n-terminal amino acid sequence (IIGGSNATSGPYAARLF) as fibrinolytic serine proteases (F-I-1 and F-I-2) from the earthworm *Lumbricus rubellus* with 100% identity (Nakamura et al., 2001). In our work, suggested that the activity of purified enzyme was rapidly decreased when the enzyme was treated with EDTA, compared with the non treatment with EDTA as show in Fig. 3. This result strongly confirmed that EDTA affected the stability of PLA-degrading enzyme. These finding also previously reported by Hadj-Ali et al. (2007) mentioned that about 70% of serine protease activity was inhibited by EDTA, indicating the involvement of any metal ion such as Ca²⁺ in dimensional structure of protease from *Bacillus licheniformis*. Because of EDTA is chelating agent, due to its ability to sequester metal ions. Thus, the metals also affected the thermal stability of the enzyme through its binding mechanism on protein that prevented unfolding at high temperature in thermophilic bacteria.

According to the report on the effect of CaCl₂ on enzyme stability at high temperature, further experiment was investigated in our work. The pure PLA-degrading enzyme was incubated with various concentrations of CaCl₂ (1, 5, 10 mM) for 30 min at various temperatures (30-100°C). Fig. 4 indicates that after treatment of the enzyme with CaCl₂, the enzyme stability decreased when compared with the control (un-treated enzyme). Moreover, increasing the concentration of CaCl₂ resulted in decrease of the stability,

indicating that CaCl_2 did not enhance the stability of enzyme but inhibited the activity of the enzyme.

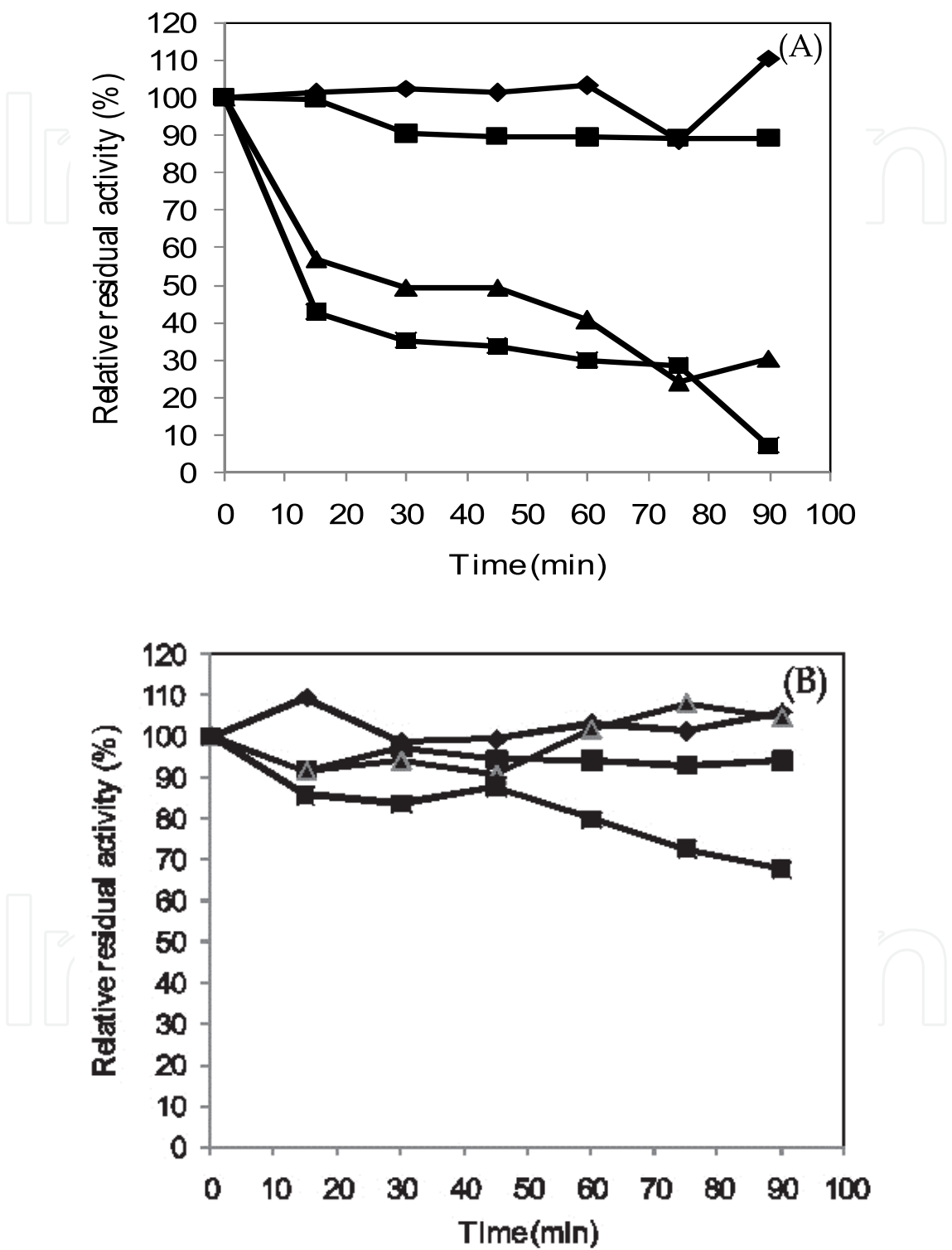


Fig. 3. Stability of purified PLA-degrading enzyme produced by *A. keratinilytica* strain T16-1 at various temperatures, 40°C (◆), 50°C (■), 60°C (▲) and 70°C (×). (A) EDTA-treated enzyme and (B) EDTA-untreated enzyme.

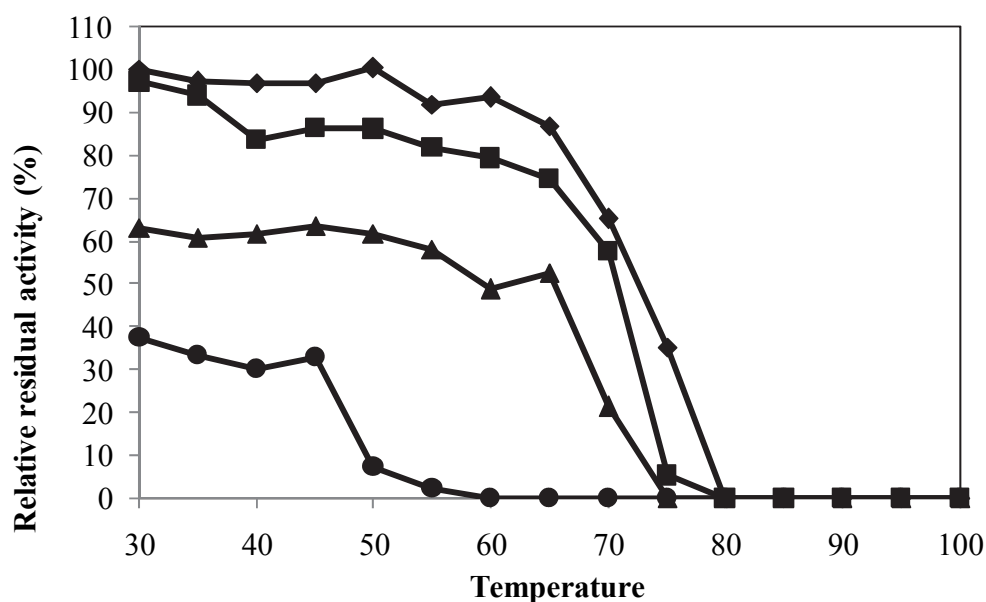


Fig. 4. The effect of CaCl_2 concentration on stability of purified PLA-degrading enzyme produced by *A. keratinilytica* strain T16-1. Without CaCl_2 (◆), 1 mM CaCl_2 (■), 5 mM CaCl_2 (▲), 10 mM CaCl_2 (●).

5. Development of fermentation process for PLA-degrading enzyme production

Factor affecting PLA-degrading enzyme production have not been studied so far. Mostly the researchers focused on the isolation and identification of new PLA-degrading microorganisms. *Amycolatopsis* sp. strain 3118 was isolated and identified by Ikura & Kudo (1999). The optimum condition for degradation of PLA film were 43°C at pH about 7.0 in a mineral salt medium with a low concentration of organic nutrients (0.002% yeast extract) and PLA film disappeared within 2 weeks. Jarerat & tokiwa (2001) demonstrated the degradation of PLA by using *T. album* ATCC 22563 found that in liquid basal medium containing PLA film, no film degradation was observed. However, by addition of 0.1% gelatin, about 76% of PLA film was degraded after 14 days of cultivation at 30°C. PLA-degrading enzyme production by *Tritirachium album*, *Lentzea waywayandensis* and *Amycolatopsis orientalis*, were inducible in the basal medium by 0.1% (w/v) poly-L-amino acids, peptides or amino acid. Silk fibroin was the best inducer for *A. orientalis* and that elastin was the best inducer for *L. waywayandensis* as well as *T. album* with the enzyme activity 450, 96 and 60 U/ml, respectively (Jarerat et al., 2004). In 2006, Jarerat et al. reported that silk fibroin was the best substrate for production of PLA-degrading enzyme in liquid medium by *Amycolatopsis orientalis* with 600 TOC formation:mg/l. The enzyme production was scaled-up in 5L jar fermenter with 550 TOC formation:mg/l after 3 days cultivation. Response surface method and central composite design were increasingly used for optimization of various phases in some fermentation process such as physical parameter and factors (temperature, pH, aeration rate, and agitation rate), fermentation medium (carbon and nitrogen sources, mineral salt and inducer) with various microorganisms. This method has been successfully applied to improve the production of many important enzyme for example α -amylase (Tanyidizi et al., 2005), xylanase (Li et al., 2007) and

keratinase (Anbu et al., 2007). However, improvement of PLA-degrading enzyme production by using response surface method has not been studied. Sukkhum et al. (2009b) demonstrated that PLA film and gelatin were found to be major effects on the enzyme production by *A. keratinilytica* strain T16-1 based on the “one factor at a time method”. The CCD experiments were designed to obtain the best condition for the maximum PLA-degrading enzyme production by the strain. The experimental design matrix and results obtained for enzymes activities were shown in Table 3. Treatment runs 9-11 were the center points in the design, which were repeated three times for estimation of error. By applying multiple regression analysis on the experimental data, the following second order polynomial equation (Eq. 1) was used to explain the enzyme production. The statistical optimal values of variables were obtained when moving along the major and minor axis of the contour, and the response at the centre point yielded maximum PLA-degrading enzyme production (Fig. 5). These observations were also verified from canonical analysis of the response surface. The canonical analysis revealed a minimum region for the model. The stationary point presenting a maximum PLA-degrading activity had the following critical values: PLA film: 0.035% (w/v) and gelatin: 0.238% (w/v). The predicted PLA-degrading activity for these conditions was 40.4 U/ml. The model was validated by repeating the experiment under the optimized conditions. The maximum experimental response for PLA-degrading enzyme production was 44.6 U/ml after 96 h cultivation with productivity of 0.46 U/ml/h. The enzyme activity obtained was 1.32 folds higher than the activity predicted by the optimized medium by one factor at a time method.

$$Y = -16.444 + (890.993X_1) + (347.718X_2) - (9004.569X_1^2) - (650.037X_2^2) - (1108.419X_1X_2) \quad (1)$$

Where Y is the predicted response (PLA-degrading enzyme production); X_1 , X_2 are coded values of PLA film and gelatin, respectively. The schematic diagrams of 3L airlift fermenter used throughout this study are shown in Fig.6. The fermentation was carried out in 3L airlift bioreactor with 2L working volume, which was 185 mm in diameter and 632 mm high. The bioreactor, which surrounded by a water jacket for temperature control, was made from glass. The air sparger was a multi porous plate (10 mm in diameter) located at the bottom of the bioreactor. The DO probe, pH probe and antifoam sensor were positioned at the top of the bioreactor. The antifoam sensor was located at 10 cm from the top of the upper broth surface. All the probes and sensor were interfaced with a control unit. The feasibility of the regression models was also carried out in a 3L airlift fermenter at aeration rate of 0.5 vvm, un-controlled pH 7.0 and 50°C. The maximum PLA-degrading enzyme production at 72 h cultivation was 150 U/ml with the productivity of 2.08 U/ml/h. A significant increase of 3.36 and 4.50 folds in PLA-degrading enzyme production and productivity, respectively, was observed in airlift fermenter.

Development of fermentation process of PLA-degrading enzyme production by strain T16-1 was summarized in Table 4. Yeast extract was used as organic nitrogen source for un-optimized medium. The enzyme activity 22 U/ml was obtained. The second step, gelatin was found to be a factor affecting the enzyme production which PLA-degrading activity 34 U/ml was obtained. The optimization of medium composition by using CCD in shake flasks was achieved which the concentration of 0.035% (w/v) PLA film and 0.24% (w/v) gelatin, with 45 U/ml of enzyme activity. At the last step of the enzyme production, the statistical

model was validated in airlift fermenter using optimized medium under the condition: aeration rate of 0.5 vvm, initial pH 7.0 (un-controlled) and temperature 50°C. The enzyme activity increased up to 150 U/ml under this condition. In conclusion, PLA-degrading enzyme production by *A. keratinilytica* strain T16-1 was significantly increased about 7 folds. We suggest that experimental design by statistical method might be useful for improvement of PLA-degrading enzyme production from other strains.

Treatment number	Level		Actual level		PLA-degrading enzyme activity (U/ml)	
	X ₁	X ₂	X ₁	X ₂	Observed	Predicted
1	1	1	0.07	0.28	22.78	25.71
2	1	-1	0.07	0.12	19.78	24.16
3	-1	1	0.03	0.28	37.52	39.08
4	-1	-1	0.03	0.12	26.90	29.92
5	1.41	0	0.10	0.20	6.74	5.11
6	0	1.41	0.05	0.40	18.93	18.51
7	-1.41	0	0	0.20	27.57	27.10
8	0	-1.41	0.05	0	7.22	5.59
9	0	0	0.05	0.20	40.73	38.05
10	0	0	0.05	0.20	40.70	38.05
11	0	0	0.05	0.20	40.47	38.05

Table 3. Experimental design used in response surface methodology of 2 independent variables, (X₁) PLA film and (X₂) gelatin, with three center points, and the observed and predicted PLA-degrading activity.

Step	Method	Condition	Enzyme activity (U/ml)
1	Un-optimized medium/condition	0.1% PLA film 0.1% (w/v) yeast extract	22
2	Screening of factors affecting the enzyme production using one factor at a time method	0.05% (w/v) PLA film 0.2% (w/v) gelatin	34
3	Optimization of medium composition using CCD in shake flasks	0.035% (w/v) PLA film 0.24% (w/v) gelatin	45
4	Validation of the model in an airlift fermenter	0.035% (w/v) PLA film 0.24% (w/v) gelatin aeration rate of 0.5 vvm Initial pH of 7.0 (un-controlled) temperature at 50°C	150

Table 4. Development of fermentation process of PLA-degrading enzyme production by *A. keratinilytica* strain T16-1

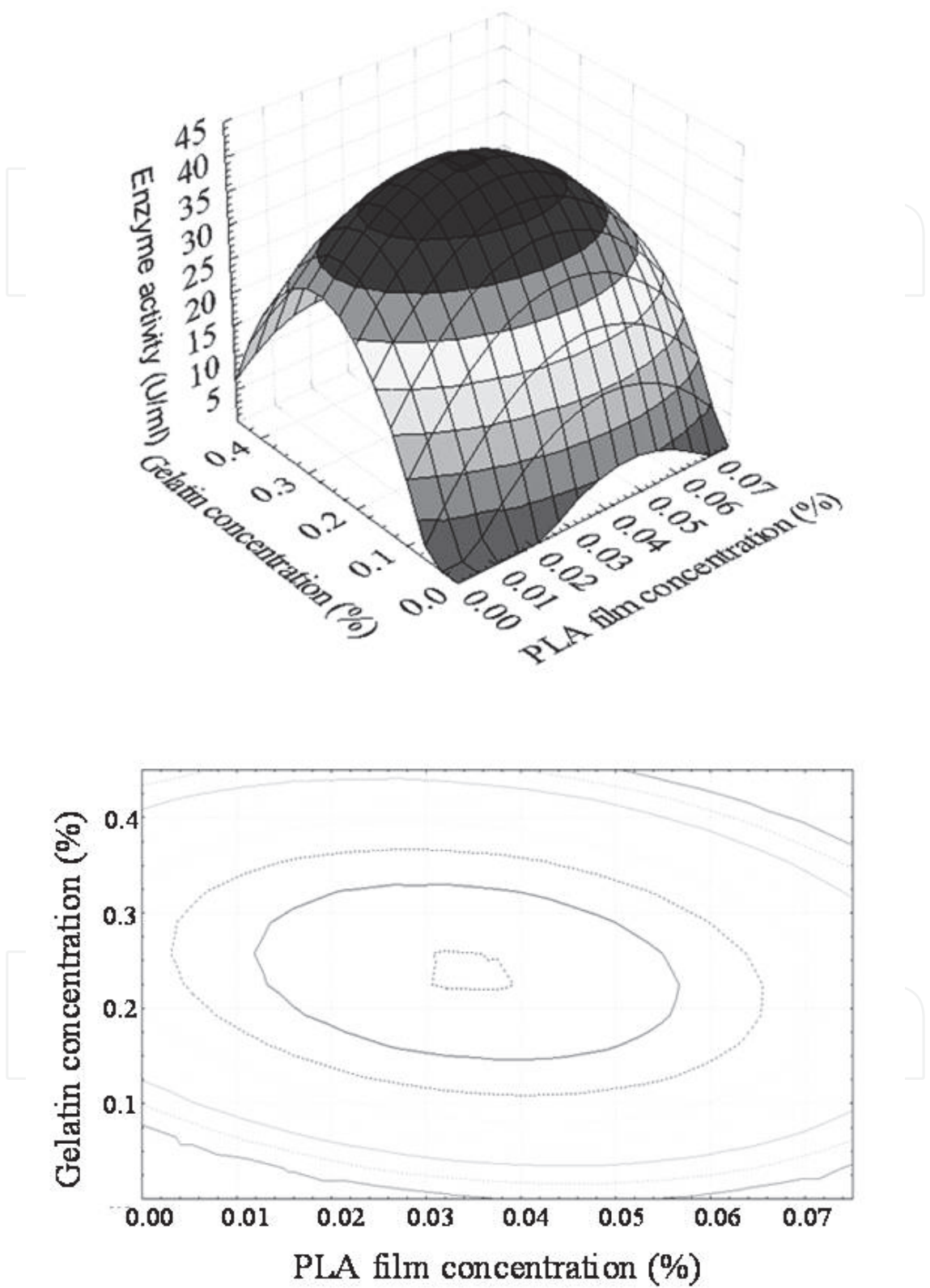


Fig. 5. Response surface described by the model, representing PLA-degrading enzyme activity (U/ml) as a function of PLA film and gelatin concentrations (Sukkhum et al., 2009b).

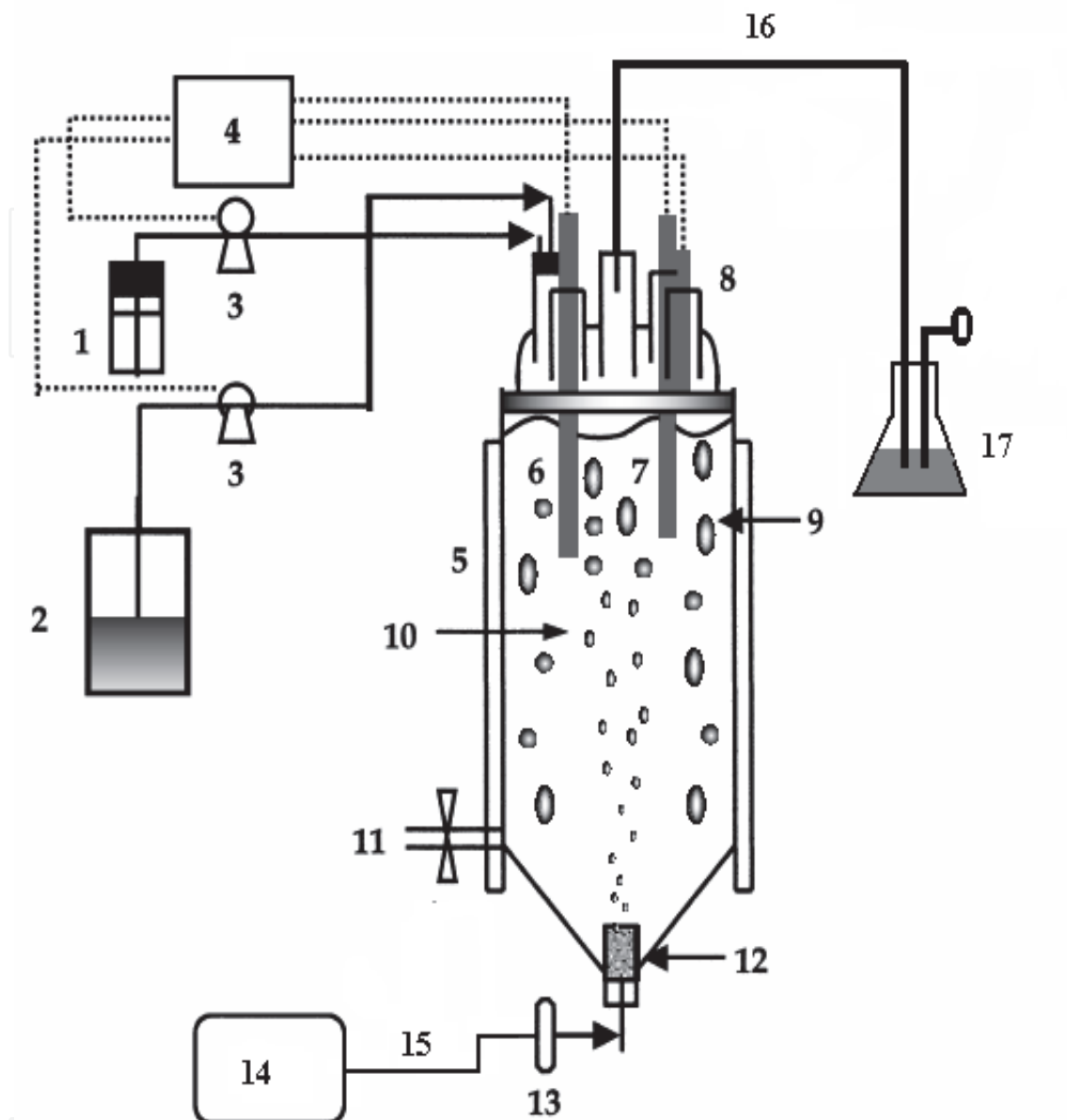


Fig. 6. Schematic diagrams of 3L airlift bioreactor used throughout this study. Antifoam, 1; Alkaline/acid reservoir, 2; Pump, 3; Controller, 4; Water jacket, 5; pH probe, 6; DO probe, 7; Antifoam sensor, 8; Dispersed bubble, 9; Bubble, 10; Sampling line, 11; Air filters (sparger), 12; Air flow meter, 13; Air pump, 14; Air inlet line, 15; Air outlet line, 16; 4% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ solution, 17.

6. Biological recycling of PLA

Currently, recycling of polymers is necessary for utilizing materials efficiently and can help to reduce the plastics wastes which effect to global worming. Methods for PLA recycling, e.g. pyrolysis (Fan et al., 2003) and chemical hydrolysis (Tsuji & Nakahara, 2002), have been reported. Poly (L-lactide) with calcium salt end structure (PLLA-Ca) is a promising material for PLLA recycling because of the ease of lactide recovery through the unzipping depolymerization process. However, the pyrolysis of PLLA-Ca also causes to form DL-

lactide at high temperature about 250°C (Fan et al, 2003). Amorphous and crystallized poly(L-lactide) (PLLA) films were prepared by quenching and annealing at 140°C for 600 min, respectively, from the melt. Their hydrolysis is investigated at pH 2.0 in HCl and DL-lactic acid (DLA) solutions (37°C) for up to 300 days to obtain hydronium ions and the lactic acid oligomers and monomers. Biological processes by both microbial and enzymatic activities are currently considered as the sustainable recycling method for polyesters. Biological recycling of PLA is one application of PLA-degrading enzyme to recycle of the plastic wastes containing PLA. The treatment process by the activity of enzyme is under mild condition, regarding as a clean process, and does not contain any undesirable by-products such as racemic of PLA after degradation. Poly(lactic acid) such as poly(DL-lactic acid), poly(D-lactic acid) and poly(L-lactic acid) were degraded by the activity of lipase in an organic solvent to produce cyclic oligomer with several enzyme for example, lipase RM (Lipozyme RM IM) and lipase CA (Novozym 435) at 60-100°C (Takahashi et al., 2004). This report suggested that the cyclic oligomer might be suitable for repolymerization and recycling of PLA. The lipase-catalyzed transformation will open a novel route for sustainable chemical recycling of polymers (Fig. 7). 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.

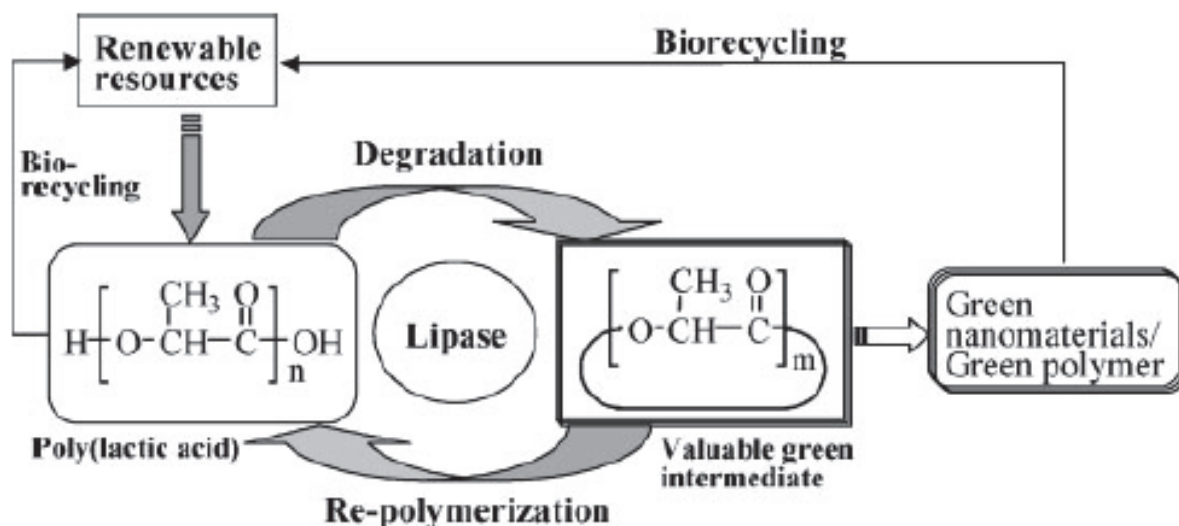


Fig. 7. Concept of poly (lactic acid) recycling (Takahashi et al., 2004)

7. Conclusion

Since 1997, many researchers have been isolated and identified PLA-degrading microorganisms. Several PLA-degrading microorganisms have been reported such as thermophilic bacteria, actinomycetes and fungi. Furthermore, PLA-degrading actinomycetes were found to distribute into various families for example *Pseudonocardiaceae*

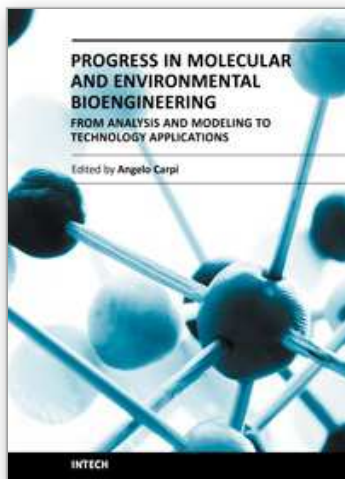
Thermomonosporaceae, *Micromonosporaceae*, *Streptosporangiaceae*, *Bacillaceae* and *Thermoactinomyces*. Molecular techniques like metagenomic library used to study the other unculturable PLA-degrading microorganisms in ecosystem. However, many other PLA-degrading microorganisms have not been isolated from the natural environment. So, further study on isolation and identification of a potent PLA-degrading strain which produced high activity should be investigated. Normally, purified PLA-degrading enzyme were characterized into two groups such as protease and lipase which showed the substrate specificity with PLA, protein, peptide and some of synthesized fatty acid at high temperature (37-70°C) and alkaline pH (9.5-10). However, the mechanisms of PLA degradation by microorganisms and enzymes should be more clearly understood by studying the enzyme purification from other microorganisms. Response surface method was successful to improve PLA-degrading enzyme production by *A. keratinolytica* strain T16-1. The optimum concentration of both PLA and gelatin as carbon and nitrogen sources, 0.035% and 0.24% (w/v) respectively, showed the maximum PLA-degrading activity was obtained by using this statistical method. The maximum PLA-degrading activity in 3L airlift fermenter with statistical optimized medium was 150 U/ml under the condition: pH 7.0 (uncontrolled), aeration rate of 0.5 vvm and 50°C. We suggest that this experimental design might be useful for improvement of PLA-degrading enzyme production from other strains. Beside, the recycling method of PLA by using enzymatic degradation was available and use mild condition without undesirable products. Recycling of biopolymer, e.g. PLA by using microbial enzyme should be achieved more details, especially in the process of biodegradation; bio-recycling and re-polymerization to open new technology for reduce the plastic wastes in the future.

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