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THESIS

PLLA-DEGRADING ENZYME PRODUCTION BY LACEYELLA SACCHARI LP175 USING AGRICULTURAL PRODUCTS AS SUBSTRATES AND ENZYME CHARACTERIZATION

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A Thesis Submitted in Partial Fulfillment of
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Srisuda Hanphakphoom 2014: PLLA-degrading Enzyme Production by *Laceyella sacchari* LP175 using Agricultural Products as Substrates and Enzyme Characterization. Doctor of Philosophy (Microbiology), Major Field: Microbiology, Department of Microbiology. Thesis Advisor: Associate Professor Vichien Kitpreechavanich, D.Eng. 119 pages.

Eleven strains of poly(L-lactide)(PLLA)-degrading thermophilic bacteria isolated from forest soils were selected based on their ability on PLLA-degrading enzyme production at 50 °C. Among the isolates, strain LP175 showed the highest PLLA-degrading ability. Strain LP175 was identified to *Laceyella sacchari* based on similarity of 99.9% similarity on 16S rRNA gene sequence and its morphological, cultural and physiological characteristics. Factors affecting PLLA-degrading enzyme production by *L. sacchari* LP175 indicated that cassava chip and soybean meal as carbon source and proteinaceous substance, respectivly yielded the highest PLLA-degrading enzyme production. The production was stimulated by phosphate and Mg^{+2}, but repressed by ammonium sulfate. The optimal concentration of cassava chip, soybean meal and PLLA powder were 0.464%, 0.153%, and 0.031%, respectively by using central composite design (CCD) in the basal medium consisting of 0.2 % K_2HPO_4, 0.1% KH_2PO_4 and 0.02% $MgSO_4.7H_2O$ The statistical model predicted PLLA-degrading activity of 68.5 U/ml and the observed value was 65.5 U/ml. The optimal physical conditions were 0.5 vvm aeration rate, temperature 50 °C and pH 7.0 for PLLA-degrading enzyme production in the 3-L airlift fermenter that increased up to 94.4 U/ml within 18 h cultivation. To our knowledge, this is the first report of a low cost medium for PLLA-degrading enzyme production by *L. sacchari* LP175.

The PLLA-degrading enzyme produced by the strain was purified to homogeneity by 48.1% yield and specific activity of 328 U/mg protein with a 15.3-fold purity increase. The purified enzyme was strongly active against specific substrates such as casein and gelatin, and weakly active against Suc-$(Ala)_3$-*p*NA. Optimum enzyme activity was exhibited at a temperature of 60 ^oC with thermal stability up to 50 ^oC and a pH of 9.0 with pH stability in a range of 8.5–10.5. Molecular weight of the enzyme was approximately 28.0 kDa, as determined by gelfiltration and SDS-PAGE. The inhibitors PMSF, EDTA, and EGTA strongly inhibited enzyme activity, but the activity was not inhibited by 1 mM 1,10-phen. The N-terminal amino acid sequences had 100% homology with thermostable serine protease (thermitase) from *Thermoactinomyces vulgaris.* The results obtained suggest that the PLLA-degrading enzyme produced by *L. sacchari* strain LP175 is serine protease.

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Student's signature Thesis Advisor's signature

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PLLA-DEGRADING ENZYME PRODUCTION BY LACEYELL SACCHARI LP175 USING AGRICULTURAL PRODUCTS AS SUBSTRATES AND ENZYME CHARACTERIZATION

INTRODUCTION

The world today develops in science and technology, increases the global population, plastic materials have been produced in many industries and used in the people living life. The most plastic materials are produced from non-biodegradable materials such as polyethylene, polypropylene, polystyrene, poly (vinyl chloride) and poly (ethylene terephthalate). The bioplastic is commercial interest for market in recent years, especially the environmental friendly plastic such as poly lactic acid. It is not only completely produced by fermentation from renewable resource (sugar crops and starch crops) but also fully biodegradable (Gupta *et al*., 2007; Jarerat *et al*., 2006; Tokiwa and Calabia, 2006; Tokiwa *et al.*, 2009).

Poly(l-lactide) (PLLA); $(C_3H_4O_2)n$ is one of the aliphatic biodegradable polyesters derived from renewable resources such as corn, cassava, sugar cane, rice and potato through lactic acid fermentation, and it is also fully degradable by both microbial and enzymatic processes (Tokiwa *et al*., 2009; Gupta *et al*., 2007; Jarerat et al., 2006; Tokiwa and Calabia, 2006; Tomita *et al*., 2004). PLLA, as an environmentaly friendly (eco-friendly or "green") product, has been developed on a large scale and is currently used for a wide range of applications, including packaging materials (Bhalla *et al*., 2007; Nolan-Itu Pty Ltd., 2002), medical applications (Jalil, 1990), agricultural products (Gross and Kalra, 2002; Sakai *et al*., 2001) and textiles.

Most PLLA-degrading microorganisms are found in the bacterial order *Actinomycetales,* e.g. the families *Pseudonocardiaceae* (Pranamuda and Tokiwa, 1999; Pranamuda *et al*., 1997), *Thermomonosporaceae* (Sangwan and Wu, 2008; Sukkhum *et al*., 2009b), and *Streptosporangiaceae* (Sukkhum *et al*., 2009b). Apart from that, thermophilic bacteria in the families *Thermoactinomycetaceae* (Sukkhum *et al*., 2009b) and *Firmicutes* (Oda *et al*., 2000; Sakai *et al*., 2001), filamentous fungi in

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the genera *Tritirachium* and *Paecilomyces* (Sangwan and Wu, 2008), and yeast in the genus *Cryptococcus* have also been reported to be PLLA-degrading microorganisms.

Many different types of enzymes are found in PLLA-degrading microorganisms, such as: protease from *Amycolatopsis* sp. strain HT-32 (Pranamuda *et al*., 1997)*, Amycolatopsis* sp. strain 3118 (Ikura and Kudo, 1999), *Tritirachium album* strain. ATCC 22563 (Jarerat and Tokiwa, 2001), *Amycolatopsis* sp. strain 41(Pranamuda et al., 2001), *Amycolatopsis* sp. strain K104-1 (Nakamura *et al*., 2001), and *Kibdelosporangium aridum* (Jarerat *et al*., 2003); serine protease from *Tritirachium album* (Williams, 1981) and *Actinomadura keratinilytica* strain T16-1 (Sukkhum *et al*., 2009b); alkaline protease from *Bacillus lentus*, *Bacillus subtilis* and *Bacillus licheniformis* (Oda et al., 2000); lipase (esterase) from *Bacillus smithii* strain PL21 (Sakai *et al*., 2001); lipase from *Paenibacillus amylolyticus* strain TB-13 (Akutsu-Shigeno et al., 2003); lipase (cutinase) from *Cryptococcus* sp. strain S-2 (Masaki *al et*., 2005); and esterase from *Geobacillus thermocatenulatus* (thermophilic) (Tomita *et al*., 2004). Furthermore, commercial enzymes from microorganisms have been reported to possess the capacity to degrade PLLA: e.g. trypsin, elastase and proteinase K, and mammalian enzymes such as α-chymotrypsin (Lim *et al*., 2005).

A few reports have investigated the purification and characterization of PLLA-degrading enzymes produced by various microorganisms: *Amycolatopsis* sp. strain K104-1 (Nakamura *et al*., 2001), *Amycolatopsis* sp. strain 41 (Pranamuda *et al*., 2001), and *Amycolatopsis orientalis* ssp. *orientalis* (Li *et al*., 2008), which belong to the family *Pseudonocardiaceae*; *Actinomadura keratinilytica* strain T16-1 in the family *Thermomonosporaceae* (Sukkhum *et al*., 2009b); thermophilic *Bacillus smithii* in the family *Bacillaceae* (Sakai *et al*., 2001); *and Paenibacillus amylolyticus* strain TB-13 in the family *Paenibacillaceae* (Akutsu-Shigeno *et al*., 2003).Because the natural degradation of PLLA in landfills, which occurs at high temperature, and the recycling process of PLLA by enzymatic method both require enzyme activity at high temperatures as well as thermostability, PLLA-degrading thermophilic strains were therefore isolated and selected. The phylogenic relationship of isolated thermophilic bacteria was also examined. As previously mentioned, the characterization of PLLA-

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degrading enzyme activity from microorganisms has been limited, including that of the selected strain, *L. sacchari* LP175, belonging to the family *Thermoactinomycetaceae*, which produced high activity. Therefore, purification and characterization of the PLLA-degrading enzyme produced by this strain was studied to determine its properties, toward the future implementation of this enzyme in the recycling of PLLA.

The various agricultural products with actinomycetes can be used to the producing PLLA-degrading enzyme. If agricultural products can be used as substrates for the microbial production, it helps reduce bioprocessing costs. The previous reports have been reported to be protease produced by microorganisns using agricultural products as substrate, such as: *Aspergillus carbonarius* (5% soybean meal) (Ire *et al.*, 2011) and *Brevibacterium linens* DSM 20158 (1.2% soybean meal) (Shabbiri *et al.*, 2012*)*. Currently, Sukkhum and coworker (2012) studied of the PLLA-degrading enzyme in an airlift fermentation by *A. keratinilytica* NBRC 104111 strain T16-1 using PLLA film (0.035%, w/v) as an inducer and gelatin (0.24 w/v) as a nitrogen source found that the enzyme production can be improved up to 257 U/ml. However, the gelatin is expensive which the expensive substrate makes them unsuitable for large-scal production.

The aims of this work were to search and identification of the PLLA-degrading thermophilic bacteria, to use agroindustrial wastes as substrates and to optimize the conditional fermentation and medium composition for producing PLLA-degrading enzyme in submerged fermentation process. This study will enhanced production of PLLA-degrading enzyme and reduced cost of producing this enzyme. In addition, comparative studies on characterization of the purified PLA-degrading enzyme produced by the selected strains were examined.

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OBJECTIVES

1. To select the highest PLLA-degrading enzyme production by the selected strain.

2. To compare the PLLA-degrading enzyme production under solid state fermentation (SSF) and submerged fermentation (SmF) from selected strain.

3. To develop fermentation processes of PLLA-degrading enzyme production by the selected strain using statistical method in shaking flasks and airlift fermenter.

4. To study factors affecting PLLA-degrading enzyme production from the selected strain.

5. To purify and characterize PLLA-degrading enzyme from the selected strain.

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LITERATURE REVIEW

Poly (L-lactide) (PLLA; (-O(CH_3)CHCO-)n) is linear aliphatic thermoplastic polyester, owing to the global utilization of plastics in large quantities, their disposal as solid waste causes harmful effects on the environment. The chemical structure of PLA is depicted in Figure 1. The polymer exists in the form of three stereoisomers: PLLA, poly(D-lactide)(PDLA) and poly(DL-lactide)(PDLLA) (Tokiwa *et al*., 2009). The development of biodegradable plastics is considered to be a product innovation that can help to resolve the problems of plastic waste because they are environmentally-friendly and completely harmless used in vessels for edible products when compared with abiotic polymer (Ikura and Kudo, 1999). Although PLA is an artificial polymer but it can be degraded by PLA-degrading enzymes from microorganisms. It is one of biodegradable plastic that can solve plastic wastes problems and can replace other petroleum based plastic materials. Furthermore, it is expected to be used widely since its cost has decreased recently. PLA is known to degrade slowly in natural soil, but it takes a long time for degradation to start. It can be hydrolyzed enzymatically (Ikura and Kudo, 1999).

The inter-relationship between biodegradable plastics and bio-based plastics is shown in Figure 2. Poly(caprolactone) (PCL), and poly (butylene succinate) (PBS) are produced from petroleum based, but they can be degraded by microorganisms. On the other hand, poly(hydroxybutyrate) (PHB), poly(lactide) (PLA) and starch blends are produced from biomass or renewable resources, and are thus biodegradable.

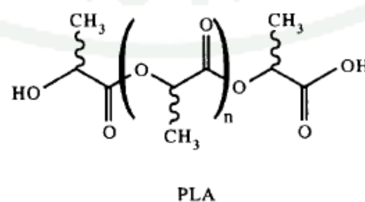

Figure 1 Chemical structure of PLA

Source: Conn *et al*. (1995)

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Despite the fact that polyethylene (PE) polymerizing ethylene gas, $H_2C{=}CH_2$ and Nylon 11 (NY11) can be produced from biomass or renewable resources, but they are non-biodegradable. Acetyl cellulose (AcC) is either biodegradable or non-biodegradable, depending on the degree of acetylation. AcC's with a low acetylation can be degraded, while those with high substitution ratios are non-biodegradable (Tokiwa *et al*., 2009).

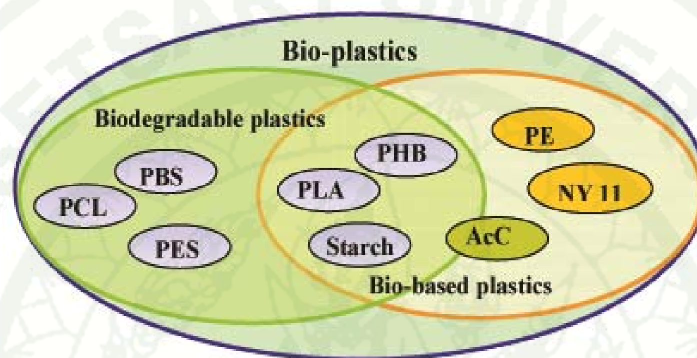

Figure 2 Bio-plastics comprised of biodegradable plastics and bio-based plastics

Source: Tokiwa *et al*. (2009)

1. PLLA production

PLA is one of biodegradable and biocompostable platics and a sustainable bioplastic to replace other petroleum-based plastic materials. The production process of PLA is shown in Figure 3. PLA can be produced from lactic acid by microbial fermentation from the annually renewable resources such as sugar crops (maizes, sugar beets, sugarcane molasses), starch crops (corn starch, wheat, sweet potatoes) and celluloses (corn stover, grasses, wheat and rice straws, and bagasses) (Nakamura *et al*., 2001, Vink *et al*., 2003; Jarerat *et al*., 2006; Tokiwa and Calabia, 2006). Lactic acid is produced through the microbial fermentation of glucose molecules in plant fibers or starch. Nowadays, the PLA synthesization from the lactic acid monomer is three major routes.

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(1) Direct condensation polymerization of lactic acid

This route involves dewatering by condensation and the use of solvent under high vacuum and temperature. The polymers produced from this route are only low- to intermediate-molecular weight material, which can be used as is, or coupled with isocyantes, epoxids or peroxide to produce a range of molecular weights.

(2) Azeotropic dehydrative condensation of lactic acid

Azeotropic dehydrative condensation of lactic acid can yield high molecular weight poly (lactic acid) without the use of chain extenders or adjustments.

(3) Polymerization through the lactide formation (or the formation of cyclic dimer of imtermediate) or formation of the cyclic dimer.

The first step of the process, the water is removed under mild conditions and without the use of a solvent to produce a low molecular weight prepolymer. This prepolymer is then catalyticall depolymerised to form a cyclic intermediate dimer called lactide and then the polymer grade is purified by using vacuum distillation. Lactide is formed by condensation of two lactic acid molecules in the three stereoisomers: poly(L-lactide) (L-PLLA), poly(D-lactide) (D-PLLA) and poly(DL-lactide or *meso*-lactide) (DL-PLLA). Constitutional unit of polylactide (PLA polymer) is formed by ring-opening polymerization (Vink *et al*., 2003, Jarerat *et al*., 2006; Gupta *et al*., 2007; Tokiwa *et al*., 2009).

Nowadays, although the PLA products are more expensive than many petroleum-derived commodity plastics, but it is inexpensive when increase the PLA products. The market and the production of PLA are greatly increasing every year because PLA have replaced petrochemical polymer. Recently, there are many plants to produce PLA on a large scale, PURAC started to build new lactide plant in 2008 in Thailand (capacity 75,000 tons lactide) and Pyramid, a new company in Germany (capacity 60,000 tons PLA in 2012). The worldwide capacity of company announcements to

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produced bioplastics are increase from 0.36 Mt in 2007 to 2.32 Mt in 2013 and to 3.45 Mt in 2020 (Figure 4) (Shen *et al*., 2010).

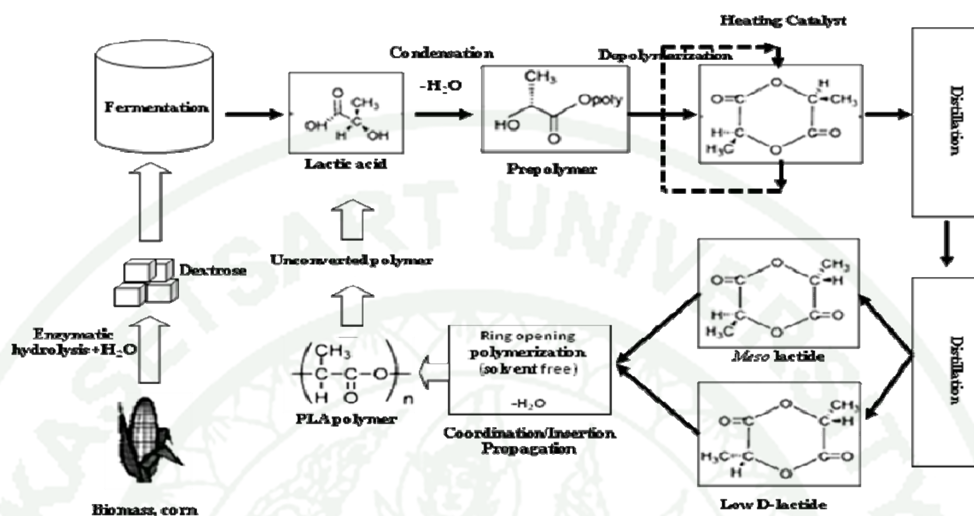

Figure 3 Current production process for poly(L-lactide)(PLA) from various renewable resources by ring opening polymerization

Source: Auras *et al*. (2004)

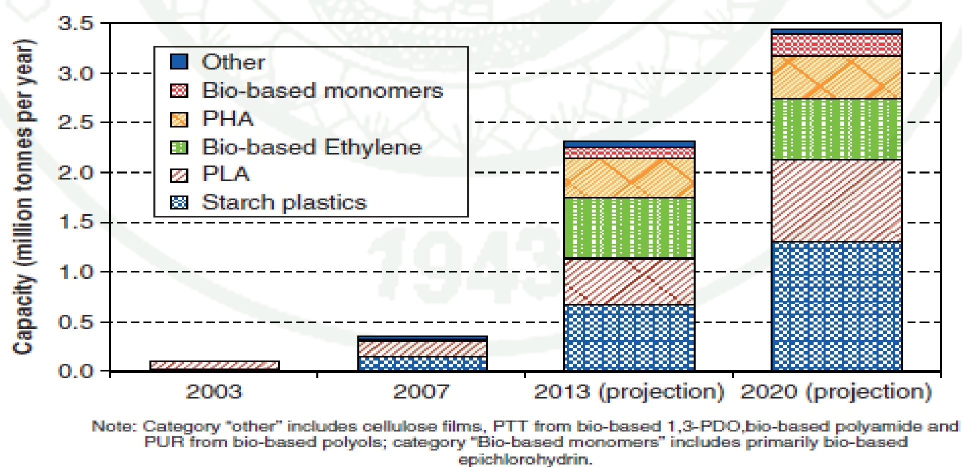

Figure 4 Woldwide capacities of biobased plastic until 2020 based on company announcements

Source: Shen *et al*., 2010

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2. Life cycle and applications of PLA

Figure 5 is shown the life cycle and application of PLA. The PLA is synthesized from latic acid which produced from farm and agricultural products (cassava, cron, corncob and potatoes) by fermentation process. The PLA is applied to many ways such as textiles, medical application and multipacks. Then, PLA materials can be recycled and composted as fertilizer in plant growth promoting.

The biodegradable plastic materials made from PLA are used in wide ranges of applications.

2.1 Packaging and containers

PLA is taken as the most promising new packaging material in the new century and it is a superstar of packaging materials related to environmental protection such as drink cups, take-away food trays, containers and planter boxes. Many PLA products are marketed under the trade name Lacea (Mitsui Toatsu, Japan), Lucty (Schimadzu, Japan) and Nature Work (Cargill Dow, USA) (Nolan-Itu Pty Ltd., 2002; Bhalla *et al*., 2007). PLA is one of most candidates for producing on a large scale by big chemical companies. Cargill Dow Ltd. Liability Co.(NatureWorks®) is the first company to producing the PLA in 2002.

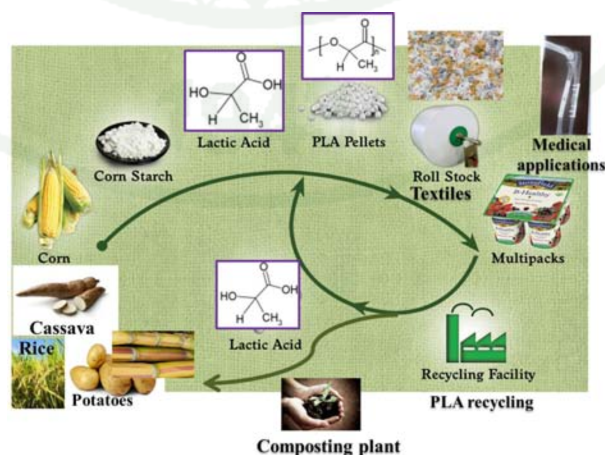

Figure 5 Life cycle of PLA

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In December 2005, NatureWorks was the primary producer of PLA (bioplastic) in the United States which produced from maize. Other companies produced PLA are Toyota (Japan), Mitsui Chemical (Lacea- Japan), Shimadzu Corporation (Japan), PURAC Biomaterials (The Netherlands), Hycail (The Netherlands), Treofan (Netherland), Galactic (Belgium), DURECT (US) and several Chinese manufacturers. The primary producer of PDLLA is PURAC, a wholly owned subsidiary of CSM located in the Netherlands. The PURAC's lactides are produced from sugar cane, potato starch and tapioca starch (Shen *et al*., 2010).

2.2 Biomedical product

PLA has researched in medical applications, such as surgical stitches, implant, drug delivery devices (Jalil, 1990). PLA is hydrolysable into soluble oligomers in the human body. It is used absorbable sutures and bone nails (degrade by trypsin). Furthermore, useful application namely the wound dressing with polylactide films was developed by the author in cooperation with physicians of the hospital "Unfallklinik Boberg". The commercialization was lunched in spring 2000 by E. Merck KGaA under the trademark Topkin® (Kricheldrof, 2001).

2.3 Agricultural product

PLA can be converted into compost that can be used to fertilizer in the soil for plant growth stimulation (Sakai *et al*. 2001; Gross and Kalra, 2002).

2.4 Textiles

PLA is expectable that it can replace typical synthesis fibers such as polyethylene, polypropylene and polystyrene.

2.5 Recycling PLA

Although some plastics are reused and recycled, but the disposal of a large quantity of used plastic waste poses a great problem. The landfill space is

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required for burying waste material, and poisonous gas is often emitted when plastic waste is incinerated. Furthermore, abiotic plastics spoil the scenery and the ecological systems of many forms of life (Ikura and Kudo, 1999). The recycling plastic can have several other advantages: 1) conservation of non-renewable fossil fuels, 2) reduced consumption of energy, 3) reduced amount of solid waste going to landfill, and 4) reduced emissions of carbon-dioxide (CO_2; save CO_2 30 to 40 % compared to conventional plastic), nitrogen-oxide (NO) and sulphur-oxide (SO_2). Normally, methods for PLA recycling, such as pyrolysis hydrolysis at a temperature higher than 200°C in water (Fan *et al*., 2003) and chemicals hydrolysis (formaldehyde, *o*-xylene, and toluene). The enzymatic hydrolysis method is an alternative method to recycle PLA because it is mild condition, regarding as clean process and does not contain any undesirable by-products such as racemic of PLA after degradation. Takahashi *et al*. (2004) were studied the recycling of PLAs (poly (D,L-,D-,and L-lactic acid) by using both enzymatic and chemical hydrolysis. The transformation into cyclic oligomer with a molecular weight of 100, the poly (D,L-lactic acid) (with a MW of 84 000) was degrade by lipase RM (Lipozyme RM IM) in a mixed solvent of chloroform/hexane at 60 ^{o}C, while the degradation of the poly (L-lactic acid) (with MW 120 000) was needed by lipase CA(Novozyme 435) at a higher temperature of 100 ^{o}C in *o*-xylene. However, recycling and reuse have limited value in solving waste management problems. Morever, the other limiting factor is that the material to be reused must maintain the quality and be no greater in cost than the virgin raw material (Jarerat *et al*., 2006).

3. Degradation and properties of PLLA-degrading enzyme

In environment, PLLA degradation occurs in two tages. The first stage, random hyhrolysis by acids and bases in high both temperature and moisture levels and cleaveage of the ester linkages in the polymer backbone leads to a reduction in molecular weight to around 40,900 Daltons. In this step occurs embrittlement of the polymers. The second stage, the molecular weight is reduced until the lactic acid and low molecule weight oligomer are naturally metabolized by microorganisms to yield carbon dioxide and water (Auras *et al*., 2004). The ester linkages in PLLA are hydrolysed by chemical reaction and cleaved by enzymatic reaction (Nolan-Itu Pty

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Ltd, 2002). Figure 6 is illustrated the scheme for the production, degradation and induction of PLA degrading enzymes. Several microorganisms i.e. *Tritirachium album (*William, 1981), *Bacillus brevis* strain 93 (Tomita *et al*., 1999), *Amycolatopsis* sp. strain K104-1 (Nakamura *et al*., 2001), *Amycolatopsis* sp. strain 41 (Pranamuda *et al*., 2001), *Bacillus smithii* (Sakai *et al*., 2001), *Paenibacillus amylolyticus* strain TB-13 (Akutsu-Shigeno *et al*., 2003), *Kibdelosporangium aridum (Jarerat et al., 2003), Saccharothix waywayandensis (Jarerat and Tokiwa, 2003a), Lentzea waywayandensis (Jarerat et al., 2004), Amycolatopsis orientalis* ssp. *orientalis (Li et al., 2008), Actinomadura keratinilytica strain T16-1 (Sukkhum et al., 2009b) and mammalian cells produce PLLA-degrading enzymes and serine proteases, respectively. These enzymes are capable of degrading PLLA and protein substrates* (silk fibroin, elastin, and gelatin), but protease degrades only protein substrates. Consequently, the degration products of microbial depolymerase (PLA-degrading enzyme and other protease) and protease, e.g. amino acids and various peptides (glycine and alanine) induce the production of PLLA-degrading enzymes (Tokiwa and Calabia, 2007). PLLA-degrading enzymes selectively clave both α-amide and α-ester bond of the L-isomer as L-alanine unit of silk fibroin (protein) (Tokiwa and Jarerat, 2004) and recognize the repeated L-lactic acid unit of PLA. The L-Ala unit of silk fibroin is one the natural analogues of L-lactate unit of PLLA (Tokiwa *et al.,* 2009).

4. PLA-degrading microorganisms

Many reports have been isolated PLA-degrading microorganisms base on clear zone method to evaluate the microbial distribution in different soil invironments. The distribution of the PLA-degrading microbes found less than PBS-, PCL- and PHB-degraging microbes. The PLA degradation takes along time for degradation to natural environment (Tokiwa *et al.,* 2009). In 1997, Pranamuda and coworkers was the first reported in the PLA-degrading microorganism *Amycolatopsis* sp., which has been isolated from soil. The phylogenic tree of PLA-degrading microoganisms is shown in Figure 7. Generally, the PLA-degradine microorganisms are distributed within family *Pseudonocardiaceae,* related genera such as *Amycolatopsis orientalis*, *Kibdelosporangium aridum*, *Streptoalloteichus*, *Saccharothrix waywayandensis*

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(rename as *Lentzae waywayandensis*) (Jarerat *et al*., 2003, Jarerat *et al.,* 2003, Jarearat and Tokiwa, 2003, Jarerat *et al*., 2004, Tokiwa and Jarerat, 2004), *Amycolatopsis* sp. strain K104-1 (Nakamura *et al*., 2001), *Amycolatopsis* sp. strain 41 (Pranamuda *et al*., 2001), and *Amycolatopsis orientalis* ssp. *orientalis* (Li *et al*., 2008). Currently, Sukkhum and coworker (2009b) found that PLA-degrading microorganisms distributed in other families of actinomyceas: family *Micromonosporaceae* consisted genus *Micromonospora viridifaciens* and *M. echinospora*; and family *Thermomonosporaceae* consisted genus *Actinomadura keratinilytica* strain T16-1; family *Streptosporangiaceae* consisted genus *Nonomuraea fastidiosa* and *N. terrinata* and other bacteria in family *Thermoactinomycetaceae* consisted genus *Laceyella sacchari* and *Thermoactinomyces vulgaris*; and family *Bacillaceae* consisted genus *Bacillus licheniformis* and thermophilic *Bacillus smithii* (Sakai *et al*., 2001). On the other hand, PLA-degrading microorganisms found distributed in family *Paenibacillaceae* consisted genus *Paenibacillus amylolyticus* strain TB-13 (Akutsu-Shigeno *et al*., 2003).

Recently studies, molecular ecological techniques were successfully used to detect and identify new PLLA-degrading uncultured microorganisms from the compost by Sangwan and Wu (2008). They are found that new information on the microbial community from family Trichocomaceae; genus *Paecilomyces* and family Thermomonosporaceae; genus *Thermomonospora* and *Thermopolyspora* .

Typical PLA-degrading enzyme consists protease and lipase (Esterase and Curtinase), which can be produced from various microorganisms as shown in Table 1.

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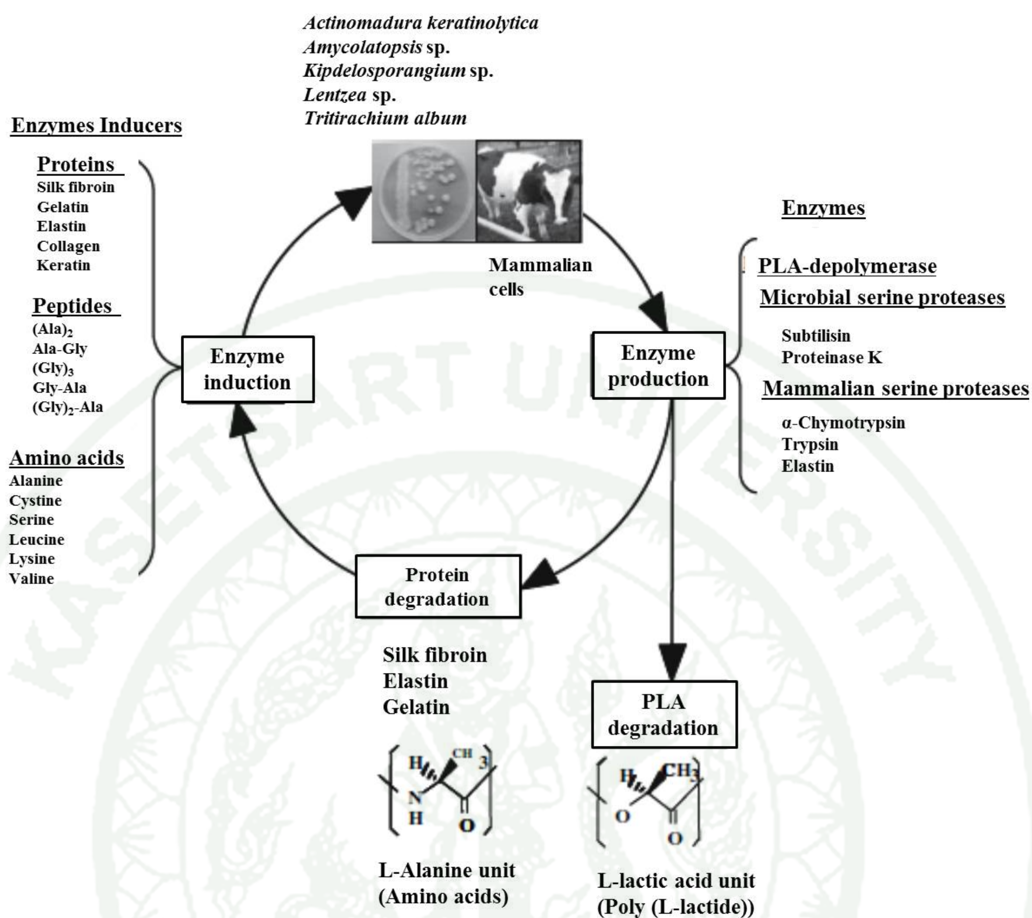

Figure 6 Scheme of the production, degradation, and induction of PLLA-degrading enzymes

Source: Modified Tokiwa and Calabia (2007)

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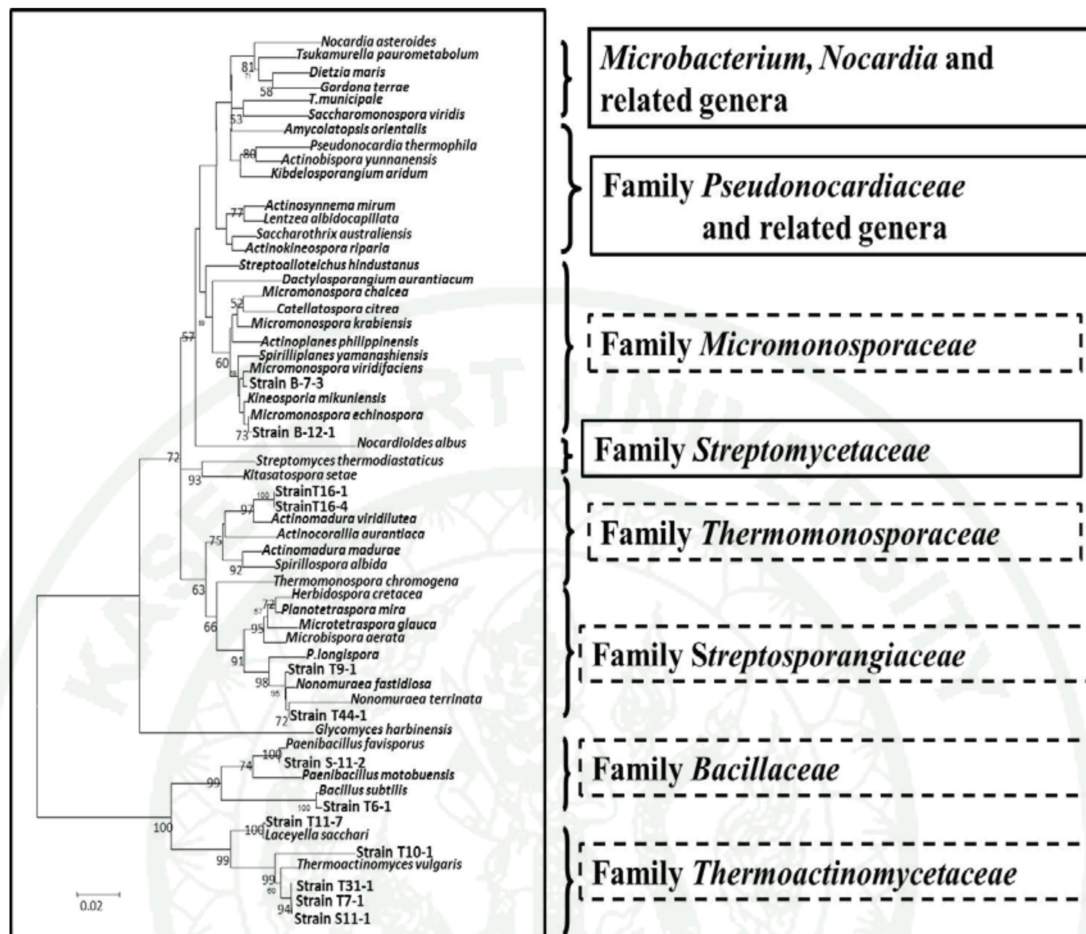

Figure 7 Phylogenetic positions of PLLA-degrading microorganisms

Source: Modified Sukkhum *et al*. (2009b)

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Table 1 Types of PLLA-degrading and commercial enzymes from microorganisms

Sources	Type of enzymes	Detection method for PLLA degradation	References
Fungi			
Tritirachium album (T. album)	Serine protease	PLLA	William (1981)
Fusarium inonoliforme	-	Film-weight loss; ceramic poly(lactic acid) oligomer production	Torres *et al.* (1996)
T. album ATCC 22563	Protease	Film-weight loss; ceramic poly(lactic acid) oligomer production	Jarerat and Tokiwa (2001)
Kibdelosporangium aridum	Protease	Film-weight loss; monomer production (lactic acid)	Jarerat *et al.* (2003)
Actinomycete			
Amycolatopsis sp. strain HT 32	Protease	Film-weight loss; monomer production (lactic acid)	Pranamuda *et al.* (1997)
Amycolatopsis sp. strain No. 3118	Protease	Film-weight loss; monomer production (lactic acid)	Ikura and Kudo (1999)
Amycolatopsis sp. strain 41	Protease	Film-weight loss; monomer production (lactic acid)	Pranamuda *et al.* (2001)
Amycolatopsis sp. strain K104-1	Protease	Turbidity method	Nakamura *et al.* (2001)
Saccharothix waywayandensis	Protease	Film-weight loss; monomer production (lactic acid)	Jarerat and Tokiwa (2003a)
Actinomadura keratinilytica strain T16-1	Serine protease	Clear zone method	Sukkhum *et al.* (2009)

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Table 1 (Continued)

Microorganisms	Type of enzyme	Detection method for PLLA degradation	Reference
Bacteria			
Bacillus brevis strain 93 (thermophilic microorganism)	Alkaline protease	Film-weight loss; monomer production (lactic acid) and viscosity	Tomita *et al.* (1999)
Bacillus smithii strain PL 21(themophilic microorganisms)	Lipase (Esterase)	Change in molecular weight	Sakai *et al.* (2001)
Panibacillus amylolyticus strain TB-13	Lipase	Turbidity method	Shigeno *et al.* (2003)
Geobacillus thermocatenulatus	Esterase	Change in molecular weight and viscosity	Tomita *et al.* (2004)
Yeast			
Cryptococcus sp. strain S-2	Lipase (Cutinase)	Turbidity method	Masaki *et al.* (2005)
Commercial enzymes			
Bacillus lentus *Bacillus subtilus* *Bacillus licheniformis*	Alkaline protease	Film-weight loss; monomer production (lactic acid)	Oda *et al.* (2000)
Pseudomonas lipase (purchased from Fluka)	Lipase	Blend film-weight loss	Lui *et al.*(2000)

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5. PLLA-degrading enzyme production by microorganisms

5.1 Carbon sources

The production of PLLA-degrading enzyme from microorganisms can be stimulated PLLA film. The best carbon source for PLLA-degrading enzyme production was PLLA film (32 U/ml). Glucose and lactose could also be used as a carbon source giving 27 and 25 U/ml enzyme activity, respectively (Sukkhum *et al*., 2009a).

5.2 Nitrogen sources

The production of PLLA-degrading enzyme from microorganisms can be stimulated several proteinous material such as silk fibroin, elastin, gelatin and some peptides and amino acids (Tokiwa *et al*., 2009; Sukkhum *et al*., 2009a).

5.3 Bath fermentation process

In spite of the fact that airlift bireactors are employed in several fermentation processes on an industrial scale, they are scarcely used as laboratory fermentors with a volume up. This is rather surprising, because small-scale airlift fermentors also have all the advantages of simplicity in construction: they are easy to make, reliable in use and low in cost. The advantage and disadvantage of airlift and stirred tank fermentation are shown in Table 2 (Anand, 2011).Besides, the PLA-degrading enzyme production was also investigated in a shaking flask and an airlift fermentor. Sukkhum and coworkers in 2009 and 2012 were developed PLA-degrading enzyme process by thermophilic *Actinomadura* keratinolytika T16-1 as shown in Table 3. In shake flasks level, the enzyme activity obtained from un-optimized medium and optimized medium composition using CCD was 33.9 and 44.6 U/ml, respectively. In an airlift fermenter, the physical factors affecting the enzyme production using CCD was succeed to improve the enzyme production up to 257 U/ml under condition at 0.43 vvm, at 46 ^{o}C and pH 6.85.

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Table 2 Comparison between an airlift and a stirred tank fermentor.

Properties	Airlift bioreactor	Stirred tank bioreactor
Investment needs	High	Low
Operating costs	High	Low
Energy requirements	Less	More
Friction	Low	High
Forming	Problem . (It can be resolve by using antifoaming agents)	No problem
Special aseptic seals	No need	Need
Scaling up	Easy	Difficult
Sterilization	Difficult	Easy
Efficiency of mixing	Low	High
Equalise shear forces	Low	High (near impeller)

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Table 3 Fermentation development for PLA-degrading enzyme by *A. keratinilytica* NBRC 104111 strain T16-1

Medium	Conditions			Enzyme activity (U/ml)	Reference
	Aeration	Temp. (^{o}C)	pH		
-0.05% (w/v) PLA film -0.2% gelatin -0.4 % $(NH_4)_2 SO_4$ -0.4 % K_2HPO_4 -0.2% KH_2PO_4 - 0.02% $MgSO_4.7H_2O$	150 rpm	50	7.0	33.9	Sukkhum *et al.*, 2009a
-0.035% (w/v) PLA film -0.24% (w/v) gelatin -0.4 % $(NH_4)_2 SO_4$ -0.4 % K_2HPO_4 -0.2% KH_2PO_4 - 0.02% $MgSO_4.7H_2O$	150 rpm	50	7.0	44.6	Sukkhum *et al.*, 2009a
-0.035% (w/v) PLA film -0.24% (w/v) gelatin -0.4 % $(NH_4)_2 SO_4$ -0.4 % K_2HPO_4 -0.2% KH_2PO_4 - 0.02% $MgSO_4.7H_2O$	0.43vvm	46	6.85	257	Sukkhum *et al.*, 2012

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6. Phylum Firmicutes

Phylum Firmicutes comprise three classes: (1) Bacilli (2) Clotridia and (3) Erysipleotrichi, but only the first two classes contain endospore-forming spesies (Figure 8).

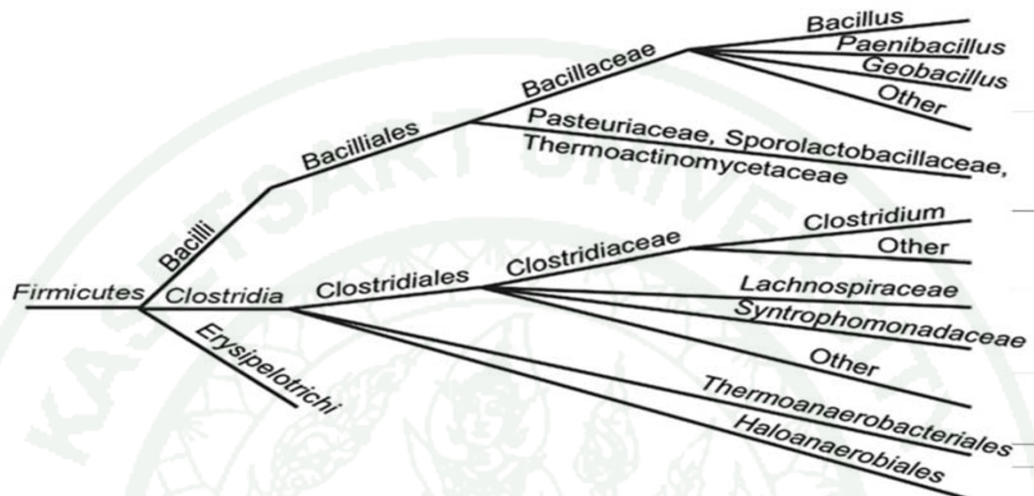

Figure 8 Phylogenetic overview of the major of endospore-forming *Firmicute.*

Source: Bueche *et al.* (2013)

The species *Laceyella* is separated as following:

Class *Bacilli*

Oder *Bacillales*

Fammily *Thermoactinomycetaceae*

Genus I. *Thermoactinomyces*

Genus II. *Laceyella*

Genus III. *Mechercharimyces*

Genus IV. *Planifilum*

Genus V. *Seinonella*

Genus VI. *Shimazuella*

Genus VII. *Thermoflavimicrobium*

Genus VIII. *Desmospora*

Genus IX. *Kroppenstedtia*

Genus X. *Melghirimyces*

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6.1 Description of *Laceyella*

Charateristics of *Laceyella* cells are aerobic, Gram-positive, non-acid-fast and chemoorganothrophic. Aerial and substrate mycelia are formed. Aerial mycerial is white. Yellow-brown or grayish-yellow soluble pigment may be produced. Sessile endospores may be produced on sporerophores. Thermophilic, the cell-wall peptidoglycan contains *meso*-DAP but no characteristic sugars. Additional characters found in this MK-9. Major fatty acids are iso-C $_{15:0}$. DNA G+C content of the type strains of two species is 48-49 mol% (determined by HCLP in this study). The type species is *Laceyell sacchari* (Yoon *et al.,* 2005). The differents of some phenotypic properties and morphological properties of genus *Laceyella* with some other genera are shown in Table 4.

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Table 4 Differential phenotypic characteristics of the genus *Laceyella* and other genera

Characteristic	*Thermoactinomyces*	*Laceyella*	*Thermoflavimicrobium*	*Seinonella*
Colour of aerial mycelium	White	White	Yellow	White
Sessile spores on dichotomously branced sporophores	-	-	+	-
Grewth on 25 μg/ml of novobiocin	+	+	+	-
Degradation of:				
Casein	+	+	+	-
Gelatin	+	+	+	-
Starch	-	+	-	-
Hypoxanthine	-	-	+	-
Xanthine	-	-	+	-
Optimal temperature for growth (^{0}C)	50-55	48-55	55	35
Predominant menaquinone	MK-7	MK-9	MK-7	MK-7
Major menaquinone (s)*	MK-8 or MK-9	MK-7 or MK-8 or MK-10	ND	MK-8, MK-9, MK-10
Major fatty acids	Iso-$C_{15:0}$; iso-$C_{17:0}$; anteiso-C15:0	Iso-$C_{15:0}$; anteiso-C15:0	Iso-$C_{15:0}$; iso-$C_{16:0}$; anteiso-C15:0	Iso-$C_{145:0}$; iso-$C_{16:0}$; anteiso-C15:0
DNA G+C content (mol%)	48	48-49	43	40

+, positive; -, negative; ND, not detected

*Other components making up > 10% peak aera ratio are shown.

Source: Yoon *et al.* (2005)

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6.2 Description of *Laceyella sachari* comb.

The description of *Laceyella sachari* is the same as that given by Lacey (1971), Lacey and Cross (1989) and Yoon *et al.* (2005) as shown in Table 5.

Table 5 Morphological and physiological characteristics of *L. sacchari*

Characteristics	*L. sacchari* (*Thermoactinomyces sacchari*)
Single spores on aerial mycelium	+
Single spores on substrate mycelium	+
Spores endospores with ridged suface	+
Spores containing dipicolinic acid	+
Spores heat-resistant	+
Spores activated by heat	+
Aerial mycelium colour	White
Mycerial walls containing *meso*-diaminopimelic acid	+
Cell sugar pattern	Type c
Mycelium septate	+
Spores on sporophores	Mostly; up to 3 µm, long
Spores sessile	Rarely
Aerial hyphae	Sparse, short, tufted
Lysis of aerial mycelium	Rapid, within 3 days
Substrate mycelium colour	Colourless to cartridge buff
Soluble pigment	Yellow-brown (some iso-lates and media only)
Assimilation of sucrose	-
Assimilation of arabinose	+
Growth on nutrient agar	Poor, thin,no aerial mycelium
Growth on yeast extract agar	Good, spreading, usually with some aerial mycelium

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Table 5 (Continued)

Characteristics	*L. sacchari* (*Thermoactinomyces sacchari*)
Glucose	++
Arabinose	+
Cellulose	-
Fructose	++
Inositol	-
Mannitol	++
Raffninose	-
Rhamnose	-
Sucrose	-
Xylose	-

growth similar to that on unamended control medium; +, more growth than on unamended control medium but less than on glucose amended medium; ++, grwth similar to that on glucose amended medium.

7. Proteolytic enzyme

Proteolytic cleavage of peptide bonds is one of the most frequent and important enzymatic modifications of proteins. Proteases are currently classified into six groups. Each family has a characteristic set of functional amino acid residues arranged in a particular configuration to form the active site (Neurath, 1989).

7.1 Serine protease

The main player in the catalytic mechanism in the chymotrypsin and subtillisin clan enzymes mentioned above is the catalytic triad. The triad is located in the active site of the enzyme, where catalysis occurs, and is preserved in all serine protease enzymes. The triad is a coordinated structure consisting of three essential

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amino acids: histidine (His 57), serine (Ser 195) (hence the name "serine protease") and aspartic acid (Asp 102). Located very near one another near the heart of the enzyme, these three key amino acids each play an essential role in the cleaving ability of the proteases. Carbohydrate sources were incorporated in nutrient agar to 1%(w/v),

7.2 Cysteine protease

The major catalytic amino acid residue is cysteine 25, acting like serine 195 in chymotrypsin (serine protease). Catalysis proceeds via a thiol ester intermediate and is facilitated by the side chains of adjacent histidine 159 and aspartic acid 158.

7.3 Aspartic protease

Aspartic protease is a family of protease enzymes that use an aspartate residue (aspartic acid 33 and 213) for catalysis of their peptide substrates. In general, they have two highly-conserved aspartates in the active site and are optimally active at acidic pH.

7.4 Metalloprotease

Metalloprotease classified by the nature of the most prominent functional group in their active site. These are proteolytic enzymes whose catalytic mechanism involves a metal. Most metalloproteases are zinc-dependent, some use cobalt. The metal ion is coordinated to the protein via two glutamic acids and one histidine imidazole ligands. The fourth coordination position is taken up by a labile water molecule.

7.5 Threonine protease and glutamic acid protease

The threonine and glutamic-acid proteases were not described until 1995 and 2004, respectively. The mechanism used to cleave a peptide bond involves making an amino acid residue that has the cysteine and threonine (peptidases) or a

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water molecule (aspartic acid, metallo- and glutamic acid peptidases) nucleophilic so that it can attack the peptide carboxyl group. One way to make a nucleophile is by a catalytic triad, where a histidine residue is used to activate serine, cysteine, or threonine as a nucleophile.

Proteolytic enzymes were classified using EC number, based on the chemical reactions they catalyze as shown in Table 6. Peptidase term is used for any enzyme that hydrolyses peptide bonds. Peptidases are recommended to be further divided into "exopeptidases" that act only near a terminus of a polypeptide chain and "endopeptidases" that act internally in polypeptide chains.

Table 6 EC number for peptidase nomenclature

Hydrolase: protease (EC 3.4)		
EC 3.4.11-19 Exopeptidase	EC 3.4.11	Aminopeptidase
	EC 3.4.13	Dipeptidase
	EC 3.4.14	Dipeptidyl-peptidase and tripeptidyl-peptidases
	EC 3.4.15	Peptidyl-dipeptidases
	EC 3.4.16	Serine-type carboxypeptidase
	EC 3.4.17	Metallocarboxypeptidases
EC 3.4.21-24 Endopeptidase	Serine endopeptidases, Cysteine endopeptidases, Aspartic endopeptidases, Metalloendopeptidases, Threonine endopeptidases	
EC 3.4.99	Endopeptidases of unknown catalytic mechanism	

Source: Moss (2007)

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8. Purification and characterization of PLLA-degrading enzyme

To date, several PLA-degrading enzymes have been reported but few have been purified and characterized. Nakamura *et al.* (2001) is the first report describing purification and characterization of a PLA depolymerase from PLA-degrading microbes (*Amycolatopsis* sp. strain K104-1). Comparison of properties of purified PLA-degrading enzymes is summarized in Table 7. The sequence of purified enzymes from *Amycolatopsis* sp. showed 40–90% homologies. The type of enzymes could also degrade PLA are proteinase K from *Tritirachium album*, cutinase-like enzyme from the yeast *Cryptococcus* sp. strain S-2, subtilisin, a microbial serine protease and some mammalian serine protease such as α-chymotrtpsn, trypsin, and lipase (elastase) (Tokiwa and Calabia, 2006). On the other hand, no homologies were observed on the sequence of the PLA-degrading serine proteases, probably because *subtilisin* and proteinase K are both microbial serine proteases while a-chymotrypsin is a kind of mammalian serine protease (Tokiwa and Calabia, 2007).

To identify and characterize PLLA-degrading enzymes are necessary because these various enzymes have different stimulates or inhibits their catalytic activity to completely degrade PLA. Moreover, if degradation of PLLA could be controlled, it can be recycled the hydrolysate as material for polymer synthesis.

9. Compost biodegradable plastics

Bidegradable plastic is plastic which will degrde from the action of naturally occurring microorganism, such as bacteria and fungi. PLA is one of the most promising bio-based plastics produced from lactic acid, it’s biodegradable and compostable. In current years, as consumers become more concerned with global warming. Commonly the ioncomplete incineration of plastic wastes (polyvinylchloride (PVC) that contain chlorinated compounds and brominated flame retardants will release hazardous doxins and furans, and increase CO_2 into the atmotsphere. The bioplastics enable a CO_2 saving of 30 to 80 % compared to conventional plastics. Composting process can reduce the burnden of incineration on local government and lead to environmental friendly method for plastics waste

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treatment. In landfill, the PLA is biodegraded and decomposted by microbial environmental which it takes a long time to degraede. Using the bacterial cells grown in substrate inoculated into composting PLA system will solve the problem (Nagao *et al*., 2008; Takahashi 2010). The composting (and biomethanization) of biodegradable plastics and biodegradable packaging is a form of recovery of waste which can cut the increasing need of new landfilling sites (Tosin *et al*. 1998).

9.1 Importance sactor for composting systems

Composting systems is a very simple process and many benefits (Takahashi, 2010):

1) To recover compost is a problem.
2) To use recycled compost unless its dualityis confirmed.
3) To reduce the cost for waste management.

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Table 7 Properties of PLLA-degrading enzyme

Type of enzyme	Source	MW (kDa)	Optimum pH	Optimum temperature	Substrate specificity	Inhibitor	N-terminal amino acid sequence	Reference
Serine type protease	*Amycolatopsis* sp. strain K104-1		9.5	55-60 °C	Casein, Fribroin	DFP, PMSF	GIGGVPASVL	Nakamura *et al.* (2001)
	Amycolatopsis sp. strain 41	43	6.0	37-45 °C	Casein, Silk fibroin, Succ-(Ala)3-pNA		GIGGVPASVK (Tokiwa and Calabia (2007))	Pranamuda *et al.* (2001)
	Bacillus smithii (thermophilic)	62.5		60 °C			NLGALPPL	Sakai *et al.* (2001)
α-chymotrypsin	Bovine pancreas				PLA, PEA, and little PBS/A		IVAGGGAVPG	Lim *et al*. (2005)
PLAase I, II and III (Serine-like proteiase)	*A.orientalis* ssp. *Orientalis*	24, 19.5, and 18	9.5, 10.9, and 9.5	50-60 °C	Casein	PMSF, Aprotinin	IVGGGTAPTVSWGAQ, IVGGGNATQVYSFMV, YDVRGGDAYYINNSS	Li *et al*. (2008)
Serine protease	*Actinomadura Keratinolytica*	30	10	70 °C	Gelatin, Suc-(Ala)3-pNA	DFP, PMSF, EDTA	GYQNNPPSAGLDRAA	Sukkhum *et al.* (2009)

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9.2 Effect affecting of composting biodegradation

The composting environment is characterized by five typical conditions: aerobiosis, solid state, high temperature, thermophilic inocula, and neutral to basic pH. Normally, the typical composting environment based on a solid state fermentation, it makes it easier to prepare "cleaner" extracts from the solid bed (Tosin *et al*., 1998). The effectors affecting to the composting biodegradation of polymer are moisture, temperature and oxygen avialabitity. According to Lotto *et al*. (2004), the temperature at 46 ^{o}C was greatest biodegraded for poly(ε-carprolactone) (PCL), poly-β-(hydroxybutyrate) (PHB), and poly-β-(hydroxybityrate-co-β -valerate) (PHB-V).

10. Solid state fermentation (SSF)

SSF is characterized by microbial growth on moist solids, has proven to be an efficient way to produce enzymes, especially by filamentous fungi, since it provides the microorganisms with the environmental akin to their natural habits (Pandey *et al*., 1999; Durand, 2003). SSF has been found potentially useful for biotechnological processes, because the substrates are cheaper than other processes. SSF is one of alternative way on the cost reduction to produce industrial enzymes. The substrates used in SSF are agro-industrial residues such as oil-mill residues (Balaji and Ebenezer, 2008).

10.1 Actinomycetes bacteria used ezymes and antibiotics productions in SSF process

A large number of microorganisms, including, yeast and fungi are ued in SSF, especially fugi such as *Aspergillus niger*, *Trichoderma* spp. and *Rhizopus* sp. The most recent reachers on SSF system have been reported for emzymes and antibiotics productions. SSF systems are used in protease production from actinomycetes such as *Actinomycetes* sp. (wheat bran as substrate) and *Streptomyces* sp. (waste hair) (Pandey *et al*., 1999).

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Normally, the commercially antibiotic productions are produced from Actinomycetes bacteria by using SmF process which it requires high energy such as and rifamycin-B (antimycobacterial) produced by *Amycolatopsis mediterranei* (Venkatrswarlu *et al*., 2000) and neomycin produced by *Streptomyces fradiae* (Ellaiah *et al*., 2004). In recent year, the researches interest in SSF process for produced commercial antibiotics. The cheapest substrates, agro-industrial residues, such as wheat bran, rice bran and other cerials appeared to be viable possibilities can be used for SFF processs.

Venkatrswarlu *et al*. (2000) reported that the comparative fermentation process for rifamicin-B production by using *Amycolatopsis mediterranei* VA18 between SSF and SmF by using wheat bran, and peanut meal and soybean with glucose as substrates, respectively. After optimization of conditions for fermentation process found that the rifamicin-B produced in SSF (90% substrate moisture, pH 7.2, 30% (v/v) inoculum size and incubation at 32 ^{o}C for 9 days) was almost 16-folds higher than that obtained in the submerged fermenter.

Ellaiah *et al*. (2004) also studied the production of neomycin by a mutant strain of *Streptomyces marinensis* NUV5 for cheaper fermentation processes with a high yield of neomycin by using SSF precess. After optimizing the process parameters such as coarse size of wheat rawa with raspberry seed powder (10% w/w) as additional nutrient, imitial moisture content of solid substrate 80 %, initial pH of medium 7.5, incubation temperature 30°C, inoculum size 0.5% w/w dry cell mass equivalent and 10 days of fermentation time, the maximum neomycin yield was increased 140 % of higher production.

10.2 Factors affecting enzyme production in SSF systems

In a SSF system, the important factors for the development of proper models are the relationships between the physiology of the microorganisms and the physic-chemicals factors. These fators affecting are consists of a suitable substrate and microorganism; pre-treatment of the substrate; particle size (inter-particle space and surface area) of the substrate; moisture content and water activity(a_w) of the

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substrate; relative humidity; type and size of the inoculum; control of temperature of fermenting matter/removal of metabolic heat; period of cultivation; maintenance of uniformity in the environment of SSF system, and the gaseous atmos-phere, i.e. oxygen consumption rate and carbon dioxide evolution rate (Pandey *et al.* 1999., Singhania *et al*., 2009).

10.3 Substrates used in SSF systems

The solid substrates are necessary to both microbial and enzyme production. The selection of substrates depends upon several factors mainly related with cost and availability and thus may invole the screening of sgro-industrial residues. The substrates have been employed for the cultivation of microorganisms containing sugar cane bagasse, wheat bran, rice bran, maize bran, gram bran, wheat straw, rice straw, rice husk, soyhull, sago hampas, grapevine trimmings dust, saw dust, corncobs, coconut coir pith, banana waste, tea waste, cassava waste, palm oil mill waste, aspen pulp, sugar beet pulp, sweet sorghum pulp, apple pomace, peanut meal, rapeseed cake, coconut oil cake, mustard oil cake, cassava flour, wheat flour, corn flour, steamed rice, steam pre-treated willow and starch (Pandey *et al*., 1999 Singhania *et al*., 2009).

11. Experimental of design

An experimental design is selected based on the objectives of the experiment and the number of factors to be investigated.

11.1 One factor of a time method (OFAT)

The traditional ‘one factor at a time” method used for optimizing a multivariable system is not only time cosuming but also often easily misses the alternative effects between compositions. Also, this method is initial step served as a screening test to identify which defined factors of the medium had a significant effect on the production. In this step beginning with a base line set of factor and measuring the response for each experimental factor in turn, the level of each factor was changed

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while holding all other experimental factors constant. For frmentation process, experimental design techniques are very useful tools for each experiments purpose which help in understanding the interactions among thr nutrient at varying concentrations and in calculating the optimal concentrations of each nutrient for a give target (i.e., maximal enzyme production). The advantagets of experimental design are the requirement of a very less number of experimentsnand thererby resulting in saving of time, glassware, chemicals and manpower, and application of a limited number fermentation process. However, this method is not very much preferred as numerous potential influential factors may be involed and their interactions could be missed (Wasli *et al*., 2009). An alternative strategy is statistical optimization, which allows rapid screening of number of factors and factor interactions, and reflects the role of each component (Zhang *et al*., 2010). A OFAT approach for optimization, the each factors was studied at different concentration levels. The approximated values were set at a middle level in the CCD for further optimization.

11.2 Response surface methodology (RSM)

Response surface methodology (RSM) is a collection of mathematical and statistical techniques for empirical model building. By careful design of *experiments*, the objective is to optimize a *response* (output variable) which is influenced by several *independent variables* (input variables). An experiment is a series of tests, called *runs*, in which changes are made in the input variables in order to identify the reasons for changes in the output response (Anonymous, d. n). RSM was created by Box and Wilson (1951) for finding the input combination that minimizes the output of a real, non-simulated system. Also see recent publications such as Meyer (1997) and Myers and Montgomery (2002) and Angűn *et al*., (2002). It is a procedure for fitting a series of regression models to the output variable of a simulation model (by evaluating it at several input variable values) and optimizing the resulting regression function (Carson and Maria, 1997).

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This methodology is to investigate the nature of the response surface over a region of interest and to identify operating conditions associated with maximum or minimum response. Generally, the operation of RSM is three phases, as emphasized in Mayer and Montgomery (2002).

Phase 0 involves the screening of explanatory variables to identify those which have a significant effect on the responses.

Phase 1 is concerned with the location of optimum operating conditions by conducting a sequence of suitable experiment.

Phase 2 involves the fitting of an appropriate empirical model, usually a second-order polynomial model, in order to examine the nature of the response surface in the vicinity of the optimum (Haines, d.n.).

For example, in the case of the optimization of the calcinations of Roman cement, the engineer wants to find the levels of temperature (x_1) and time (x_2) that maximize the early age strength (y) of the cement. The early age strength is a function of the levels of temperature and time, as follows:

$$y = f(x_1, x_2) + \varepsilon$$

where ε represents the noise or error observed in the response y. The surface represented by $f(x_1, x_2)$ is called a response surface.

The response can be represented graphically, either in the three-dimensional pace or as contour plots that help visualize the shape of the response surface. Contours are curves of constant response drawn in the x_i, x_j plane keeping all other variables fixed. Each contour corresponds to a particular height of the response surface, as shown in Figure 9 (Anonymous, d. n).

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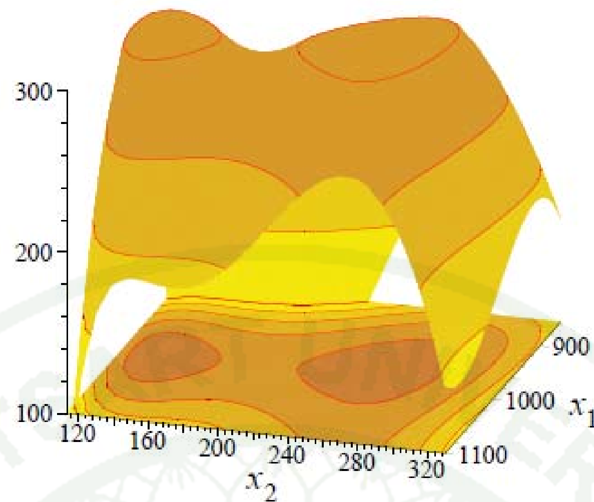

Figure 9 Three-dimensional response surface and the corresponding contour plot for the early age strength of Roman cement where x_1 is the calcinations temperature (^oC) and x_2 is the residence time (mins)

Source: Anonymous, d. n

The application of RSM to design optimization is aimed at reducing the cost of expensive analysis methods and their associated numerical noise (Anonymous, d. n). RSM is the one suitable for identifying the effect of individual variables and for seeking the optimum conditions for a multivariable system efficiently. This method has been successfully applied to optimize other production and fermentation media. Basically, this optimization process involes three major steps: 1) performing the statistically designed experiments, 2) estimaing the coefficients in a mathematical model and 3) predicting the response and checking the adequacy of the model. Ferthermore, the authors report the application of the RSM using the Box-Wilson design (Bandaru *et al*., 2006).

RSM was already applied in other field of research and is well suited with the study of the main and interaction of the factor in bioconversion yield. It is a concise way of describing and predicting response of a system of variables (Wasli *et al*., 2009).

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11.3 Central composite design (CCD)

A second-order model can be constructed efficiently with central composite designs (CCD). CCD is first-order (2*N*) designs augmented by additional centre and axial points to allow estimation of the tuning parameters of a second-order model for 3 design variables.

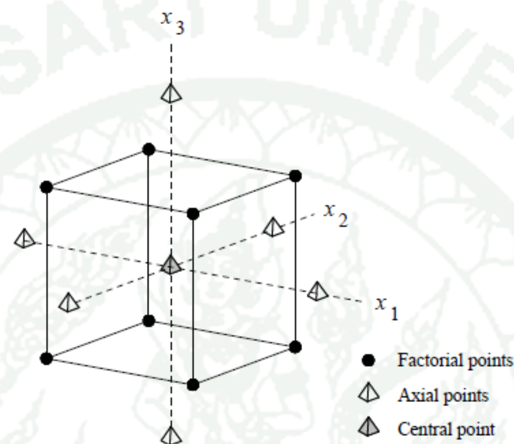

Figure 10 Central composite design for 3 design variables at 2 l evels

Source: (Anonymous, d. n)

As shown in Figure 10, the design involves 2*N* factorial points, 2*N* axial points and 1 central point. CCD presents an alternative to 3*N* designs in the construction ofsecond-order models because the number of experiments is reduced as compared to a full factorial design (15 in the case of CCD compared to 27 for a full-factorial design). CCD have been used by Eschenauer and Mistree (1997) for the multiobjective design of a flywheel (Anonymous, d. n).

In the case of problems with a large number of designs variables, the experiments may be time-consuming even with the use of CCD.

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11.3.1 Determining α in Central Composite Designs

To maintain rotatability, the value of α depends on the number of experimental runs in the factorial portion of the central composite design (Table 8):

$$\alpha = [\text{number of factorial runs}]^{1/4}$$

If the factorial is a full factorial, then

$$\alpha = [2^k]^{1/4}$$

However, the factorial portion can also be a fractional factorial design of resolution V.

The total number of experimental combinations is $2^k + 2k + n_0$, where k is the number of independent variables and n_0 is the number of repetitions of the experiments at the centre point. For statistical calculation, the experimental variables X_i have been coded as x_i according to the following transformation equation:

$$x_i = \frac{X_i - X_0}{\delta X}$$

where x_i is the dimensionless coded value of the variable X_i, X_0 is the value of X_i at the center point, and δX is the step change.

This methodology allows the modeling of a second-order equation that describes the process, The production was analyzed by multiple regression through the least squares method to fit the following equation (Xu *et al*, 2009):

$$Y = a_o + \sum a_i x_i + \sum a_{ii} x_i^2 + \sum a_{ij} x_i x_j, \ldots$$

where Y represents the predicted response variable, a_o is the interception coefficient, a_i is the coefficient of the linear effect, a_{ii} is the coefficient of

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quadratic effect and a_{ij} is the coefficient of interaction effect, xi and xj denote the code levels of variable Xi and Xj in experiments.

The statistical significance of the third-order medel equation was determinded by a significant F-value, an insignificant lack-of-fit F-value and a good multiple correlation coefficient, R^2 value.

However, the application of statistical methods to optimize the fermentation conditions and medium composition for PLA-degrading enzyme production by actinomycetes strain under SSF process has not been studies so far.

Table 8 Illustrates some typical values of α as a function of the number of factors

Number of factors	Factorial portion	Scaled value for α relative to ±1
2	2^2	$2^{2/4} = 1.414$
3	2^3	$2^{3/4} = 1.682$
4	2^4	$2^{4/4} = 2.000$
5	2^{5-1}	$2^{4/4} = 2.000$
5	2^5	$2^{5/4} = 2.378$
6	2^{6-1}	$2^{5/4} = 2.378$
6	2^6	$2^{6/4} = 2.828$

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MATERIALS AND METHODS

1. Selection of a potent of PLLA-degrading filamentous thermophilic bacteria

1.1 Bacterial strains

Eleven bacterial strains isolated from forest soils in Thailand by Miss Narisara Maneewong (Master degree in Microbiology, Kasetsart University, 2014) were used in this study (Table 9).

Table 9 PLLA-degrading isolates form forest soil samples in Thailand

Sampling location	Isolates
Chaiyaphum province	C77
	C79
	C86
	C88
	C89
Kanchanaburi province	K13
	K23
	K25
	KT176
Phetchaburi province	P43
Lampang province	LP175

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1.2 Inoculum of preparation

One loop of each isolate taken from each working stock on a NA plate was inoculated in 50 ml of nutrient broth (NB) and cultivated for 1 day in a rotary shaker at 150 rpm and 50 ^{o}C. The composition of the NB medium was (per L) 3 g beef extract, 5 g peptone (pH 7.0), and 15 g agar (NA medium). The culture was centrifuged at 10,000 rpm for 10 min (4 ^{o}C). The obtained cell pellet was then washed with sterile 0.85 % (w/v) NaCl. After that, the optical density of the cell suspension was adjusted by 0.85 % NaCl to an optical density of 0.5 at a wavelength of 600 nm, and then inoculated at 10 % (w/v) into basal medium.

1.3 Cultivation

The composition of medium (pH 7.0) was as follows (g/L): 4 g $(NH_4)_2SO_4$; 2 g K_2HPO_4; 1 g KH_2PO_4; 0.5 g $MgSO_4 \cdot 7H_2O$; 1 g yeast extract; and 1g PLLA powder (Nature work®, 100% PLLA), and after that the medium was sterilized in an autoclave at 121°C for 15 min. The PLLA powder was used as a substrate for enzyme production. Ten percents of inoculum medium were used to inoculate in this medium. Cultivation was carried out at 50 ^{o}C in a rotary shaker at 150 rpm for 3 days. The culture supernatant was harvested by centrifugation at 10,000 rpm for 10 min (4 ^{o}C) for assaying PLLA-degrading activity. The isolate which had highest enzyme activity was selected for the next step.

2. Identification of the selected PLLA-degrading producing strain

The selected strain was identified base on 16S rRNA by Nrissara Maneewong and constructed a phylogenetic tree by the two-parameter method (Kimura, 1980) and a neighbor-joining method (Saitou and Nei, 1987) using the MEGA 4 program (Kumar *et al*., 2008).

The general inocula for all phenotypic studies and special washed inocula for a carbon utilization test were prepared using the method of Shirling and Gottlieb (1996). Samples in all phenotypic studies were incubated at 50 ^{o}C for 3–5 days,

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except for the growth temperature test. Cultural characteristics of colonies were observed on NA in Petri dishes, as described by Lacey (1971). The selected strains were examined for the ability to grow at different temperatures (35–65 °C) and NaCl concentrations (0–5 %) on NA. Carbon utilization was tested on NA with 1% w/v of each carbon source (Lacey, 1971). The ability to degrade various substrates was determined using the media and methods described by Kurup et al. (1975). The aerial mycelium spores were observed under a scanning electron microscope (SU8020 Model) (Hitachi High-Technologies Ltd., Tokyo, Japan).

3. Factor affecting for PLLA-degrading enzyme production in production medium by the selected strain

3.1 Inoculum preparation

A loopfull taken from a working stock 50 ml of NA medium in 250-ml flask and incubated at 50 ^{o}C for 1 day on a rotary shaker incubator at 150 rpm. The 50 ml of seed medium was centrifuged at 10,000 rpm for 10 min and washed cell once times with steriled of 0.45 % NaCl. After washed cell, the pellet was dissolved in steriled of 0.45 % NaCl and adjusted to an OD600 nm of 0.5. This suspension of cells was used as inoculum in culture fementation.

3.2 Cultivation

Culture medium used for PLLA-degrading enzyme production in SmF contained 0.2 % K_2HPO_4; 0.1% KH_2PO_4; 0.02 % MgSO4·7H2O; 0.15 % cassava chip; 0.26 % soybean meal and 0.035 % PLLA-powder (pH 7.0). The production medium was pored in 250-ml flask and then autoclaved at 121 ^{o}C, 15 psi for 15 min. The production medium was inoculated with 10 % inoculum and incubated at 50 ^{o}C at 150 rpm in a incubator shaker. The supernatant obtained from centrifugation at 4°C was assayed PLLA-degrading activity.

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3.3 Effect of proteinaceous substances on PLLA-degrading enzyme production

To study on various proteinaceous substances on PLLA-degrading enzyme production, the basal medium with PLLA powder mixed different nitrogen sources such casamino acid,casein, gelatin, peptone tryptone, yeast extract and soybean meal were prepared and added to production medium with 3.68 % of final total nitrogen. The culture flasks were incubated at 50 ^{o}C for 2 days.

3.4 Effect of carbon sources on PLLA-degrading enzyme production

To study on various cabon sources on PLLA-degrading enzyme production, the basal medium with PLLA powder mixed different nitrogen sources. Various carbon sources were added to the production medium, with a final total carbon to 6 % (w/v) of each crbon source such as lactose, glucose, sucrose, galactose, fructose, xylose, lactic acid, PLLA. The culture flasks were incubated at 50 ^{o}C for 2 days.

4. Optimization of fermentation process for PLLA-degrading enzyme production from *L. sacchari* LP175 using agricultural product as substrates

4.1 Comparison of PLLA-degrading enzyme production in solid state fermentation (SSF) and submerged fermentation (SmF)

The *L. sacchri* LP175 can be grown on some agricultural products such as wheat bran, cassava chip and soybean meal, which is cheapest substrates for culturing microorganisms. In this work we focused on the production of PLLA-degrading enzyme in solid state fermentation and submerged fermentation by *L. sacchri* LP175 using low cost substrates.

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4.1.1 Solid state fermentation studies

(1) Inoculum preparation

The selected sttrain was grown in NA slant and incubated at 50 ^{o}C for 3 days. The culture in NA slant was added 5 mL of a Tween 80 solution (0.05 % v/v). The suspension's spore concentration was adjusted with sterile water to absorbance of an OD600 nm of 1.0 in a UV spectrophotometer (Soares *et al*., 2007). This suspension of cells was used as inoculum for SSF.

(2) Cultivation

The three agroindustrial wastes (wheat bran, soybean meal and cassava chip) were used as the solid substrate in this experiment. The twenty grams of each substrate was put in a plastic bag (8 X 12 inch). The moisture content of the substrate on PLLA-degrading production was observed at moisture content at 60 % (v/w) and then sterilized at 121 ^{o}C, 15 psi for 15 min. The mixture medium was inoculated with 10% (v/w) inoculum and then incubated 50 ^{o}C for 5 day. The mixed substrates on PLLA-degraing enzyme were evalued according to obtain 7 experiments as presented in Table 10.

(3) Seperation of crude enzyme extraction

The fermented substrate was soaked with 100 mM Tri-HCl (pH9.0) (1:2 w/v) at 4 oC on over night. The crude enzyme extract was filtrated by a Whatamn No.1 analytical filter and the filtrate obtained was centrifuged at 10,000 rpm for 10 min at 4 oC. The crude extract enzyme was assayed PLLA-degrading activity.

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4.1.2 Submerged fermentation

(1) Inoculum preparation

Preparation as follow 3.1 and 3.2

Table 10 Experiments of three agricultural products mixture for PLLA-degrading enzyme production under SSF

Experiment	Wheat bran (g/20g)	Soybean meal (g/20g)	Cassava chip (g/20g)
1	20	0	0
2	0	20	0
3	0	0	20
4	10	10	0
5	10	0	10
6	0	10	10
7	6.67	6.67	6.67

(2) Inoculum preparation

Culture medium used for PLLA-degrading enzyme production in SmF contained (g/l): 10% cassava chip; 10 % soybean meal and 0.035 % PLLA-powder. The production medium was pored in 250-ml flask and then autoclaved at 121 ^{o}C, 15 psi for 15 min. The production medium was inoculated with 10 % inoculum and incubated at 50 ^{o}C at 150 rpm in a incubator shaker. The supernatant obtained from centrifugation at 4°C was assayed PLLA-degrading activity.

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4.2 Optimization of fermentation process for PLLA-degrading enzyme production in submerged fermentation

4.2.1 Inoculum preparation

Preparation as follow 3.1

4.2.2 Effect of the basal medium composition sources on PLLA-degrading enzyme production

Cassava chip and soybean meal were selected as the source of carbon and nitrogen, respectively. PLLA powder was sdded 0.035 % in each medium. The basal medium compositions were stuied as shown in Table 11.

Table 11 Different medium formoulas (g/l) for produced PLLA-dergading enzyme by the selected strain

Medium	$(NH_4)_2SO_4$	K_2HPO_4	KH_2PO_4	$MgSO_4.7H_2O$	Cassava chip	Soybean meal	PLLA powder
1	4	2	1	0.2	1.5	2.6	0.35
2	4	2	1	0	1.5	2.6	0.35
3	4	0	0	0.2	1.5	2.6	0.35
4	0	2	1	0.2	1.5	2.6	0.35
5	4	0	0	0	1.5	2.6	0.35
6	0	2	1	0	1.5	2.6	0.35
7	0	0	0	0.2	1.5	2.6	0.35

4.2.3 Effect of initial pH sources on PLLA-degrading enzyme production

To study on the effect of pH values on the enzyme production, the initial pH of production medium was adjusted to 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0. The culture flasks were carried out at 50 ^oC for 2 days.

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4.2.4 Factors affecting PLLA-degrading enzyme production by statistical design

4.2.4.1 CCD for ingradient optimization

Based on the results from “one fector of a time”, The significant parameters of variable on PLLA-degrading activiry were estimated based on comparison of the difference in the mean between the high level (+) and the low level (-). A central composition design (CCD) was used for optimizing independent variables at each of five levels. Low and high factor setting were coded as -1 and +1 repectively, the centre point was coded as 0 and the design was extend up to +α and –α. The mathematical model.SPSS for windows and Statistica 5.0 software (Statsoft, USA) were used for experimental design, data analysis and quadratic model building. The response surface methodology (RSM) was represented by ploting contour graps from the optimal concentrations of factors obtained by a numerical optimization procedure.

To maintain rotatability for usual CCD, the value of α depends on the number of experimental runs in the factorial portion of the central composite design:

$$\alpha = [\text{number of factorial runs}]^{1/4}$$

If the factorial is a full factorial, in this study k = 3 factors (cassava chip, soybean meal and PLLA powder) could be written as Eq. (1):

$$\alpha = [2^k]^{1/4} = 1.68 \qquad (1)$$

The independent valiable range and experimental design are shown in Table 12 and 13, respectively. The independent variables were cassava chip (X_1), soybean meal (X_2) and PLLA powder (X_3). The methodology allows the modeling of “a second-order equation” that analyzed by multiple regressions through the least squares method following Eq. (2):

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$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + a_{11}X^2_1 + a_{22}X^2_2 + a_{33}X^2_3 \qquad (2)$$

where Y is the predicted response (PLLA-degrading ezyme yield); X_1, X_2 and X_3 are coded forms of the input variables as cassava chip, soybean meal and PLLA powder, respectively; a_0 is a constant; a_1, a_2 and a_3 are the linear coefficients; a_{12}, a_{12} and a_{13} are a cross-product coefficient; a_{11}, a_{22} and a_{33} are the quadratic coefficients. The relation between the coded forms of the input variable and the actual value of cassava chip, soybean meal and PLLA powder are described as Eq. (3):

$$x_i = \frac{X_i - X_0}{\delta X} \qquad (3)$$

Where x_i is the dimensionless coded value of the variable X_i, X_0 is the value of X_i at the center point, and δX is the step change (e.g., coded casein, +1 is equal to 0. 7). The center point in this study was obtained from one factor at a time experiment.

Table 12 Levels of different process variables in code and un-code form for PLLA-degrading enzyme production independent variables range and levels

Independent variables	Levels				
	-1.68	-1	0	1	1.68
Cassava chip (X_1), g/l	2.25	3.0	4.5	6.0	7.02
Soybean meal (X_2), g/l	0.66	1.0	1.5	2.0	2.34
PLLA powder (X_3), g/l	0.05	0.15	0.3	0.45	0.55

The data from the experimental design were subjected to a second-order multiple regression analysis using the least squares regression

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methodology to obtain the parameter estimators of the mathematical model. SPSS for windows and Statistica 5.0 software (Statsoft, USA) were used for regression analysis and graphical analysis of the data, respectively.

Table 13 Experimental design used in response surface methodology of 3 independent variables, (X_1) cassava chip, (X_2) soybean meal and (X_3) PLLA powder, with three center points

Treatment Number	Code setting level			Actual level		
	X_1	X_2	X_3	X_1	X_2	X_3
1	-1	-1	-1	3.0	1.0	0.15
2	-1	-1	1	3.0	1.0	0.45
3	-1	1	-1	3.0	2.0	0.15
4	-1	1	1	3.0	2.0	0.45
5	1	-1	-1	6.0	1.0	0.15
6	1	-1	1	6.0	1.0	0.45
7	1	1	-1	6.0	2.0	0.15
8	1	1	1	6.0	2.0	0.45
9	-1.68	0	0	2.25	1.5	0.3
10	1.68	0	0	7.02	1.5	0.3
11	0	-1.68	0	4.5	0.66	0.3
12	0	1.68	0	4.5	2.34	0.3
13	0	0	-1.68	4.5	1.5	0.05
14	0	0	1.68	4.5	1.5	0.55
15	0	0	0	4.5	1.5	0.3
16	0	0	0	4.5	1.5	0.3
17	0	0	0	4.5	1.5	0.3

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4.2.5 Physical factors affecting PLA-degrading enzyme production in an airlift fermenter

Schematic diagrams of airlift bioreactor shown in Figure 11. The fermentation was carried out in 3 L airlift bioreactor with 2 L working volume, which was 185 mm in diameter and 632 mm high. The temperature of bioreactor was controlled by a water jacket. The air sparger was a multi porous plate (10 mm in diameter) located at the bottom of the bioreactor and maintained at 50 ^{o}C by water bath. The DO probe, pH probe, antifoam sensor and air out were positioned at the top of the bioreactor. All the probes and sensor were controlled with a control unit. During fermentation, samples were collected from fermenter at certain time intervals for further analysis.

The inoculum and medium were prepared as follow 5.1(1) and 5.1(2). The culture was sampled every 6 h for 36 h and used PLLA-degrading enzyme activity. Althoug *L. sacchari* strain LP175 is also filamentous bacteria, but not formed pellet in the liquid medium. So, the growth was determined by drop plate method.

4.2.5.1 Effect of aeration rate on PLLA-degrading enzyme production

To study of the effect of aeration rate, air flow meter was set at 0.25, 0.5 and 0.75 vvm and maintained at 50 ^{o}C and pH 7.0. The samples were taken every 24 h for 120 h and used for degrading activity and vialble cell count assay.

4.2.5.2 Effect of temperature on PLLA-degrading enzyme production

Effects of various temperatures on the enzyme production in an airlift fermenter were examined at 40 ^{o}C and 50 ^{o}C. The aeration rate (0.5 vvm) and pH value (7.0) were fixed. The sample was taken every 6 h for 36-42 h. Each sample was used for assay PLLA-degrading activity and vialble cell count assay.

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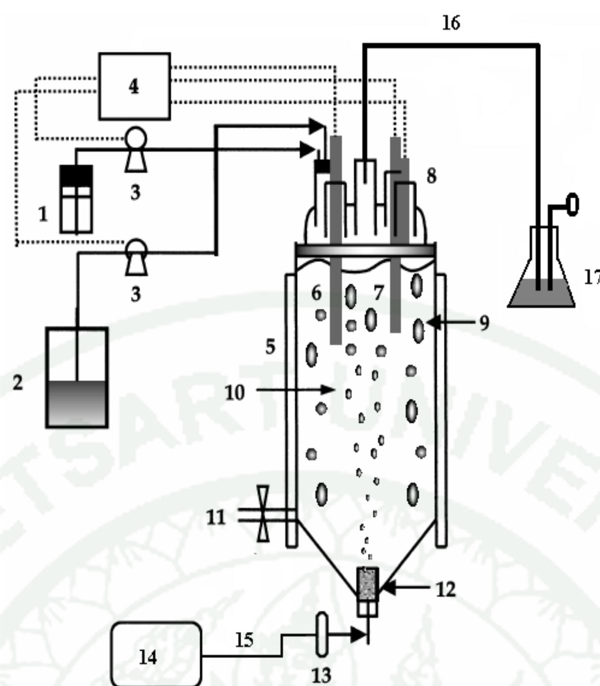

Figure 11 Schematic diagrams of 3 L airlift bioreactor used throughout this study

Experiment apparatus:

1.antifoam sensor	2. alkaline reservoir	3. pump
4. packed controller	5. water jacket	6. pH probe
7. DO probe	8. antifoam probe	9. dispersed bubble
10. sampling line	11. sparger	12. air filter
13. flow meter	14. air pump	15. air inlet line
16. air outlet line	17. 4% $CuSO_4.5H_2O$ solution	

Source: Modified from Miura *et al.* (2004)

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5. Purification and characterization of the PLLA-degrading enzymes

L. sacchari LP175 was cultured on production medium (Sukkhum *et al*. 2009a) for 2 days at 50 ^{o}C.

5.1 Purification of PLLA-degrading enzyme

Unless otherwise stated, all procedures were carried out at 4 ^{o}C. The supernatant obtained by centrifugation of the cultured *L. sacchari* LP175 was dialyzed against 20 mM acetate buffer (pH 5.0) overnight. After dialysis, the solution was loaded onto a 50 ml CM Sepharose column (5 × 30 cm) which had been previously equilibrated with 20 mM acetate buffer (pH 5.0). The column was washed with the same buffer (using twice the volume of the column), and the column was then eluted with a linear gradient of 20 mM of the same buffer containing 0 to 0.6 M NaCl solution at a flow rate of 1.0 ml/min. The each fraction was determined enzyme activity and protein concentration. The active fractions that contained PLLA-degrading activity were pooled and dialyzed with Tris-HCl buffer (pH 9.0). The pooled samples were checked for purity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

5.2 Characterization of purified enzyme

The characterizations of purified enzyme were studied using 50 μg/ml of protein.

5.2.1 Determination of optimum pH and temperature on purified enzyme activity

To test the optimal pH of the purified enzyme, the emulsified PLLA substrate was adjusted to OD 1.0 at 630 nm with 100 mM buffer solution at pH values ranging from 3.5 to 12. Four types of buffer solution were used: acetate buffer (pH 3.5–6.0); phosphate buffer (pH 6.0–8.0); Tris-HCl buffer (8.0–10.0); and glycine-NaOH buffer (pH 10.0–12.0). The effect of temperature on purified enzyme was studied at temperatures ranging from 30–100 ^{o}C. After adjusting for pH and

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temperature, the residual enzyme activity was assayed under the conditions given in the standard enzyme assay procedure.

5.2.2 Determination of stability of pH and temperature on purified enzyme activity

The pH stability of purified enzyme was tested by incubating the enzyme solution at 4 ^{o}C for 24 h in buffer solutions with pH values in the range of 3.5–12.0. The thermal stability of the enzyme was checked by incubating the enzyme for 1h at different temperatures ranging from 30–100 ^{o}C. The residual activity of the purified enzyme was assayed under optimal conditions.

5.2.3 Substrates specificity of the PLA-degrading activity on purified enzyme activity

Emulsions of 0.1% poly-(ε-caprolactone) (PCL) and olive oil were diluted with 100 mM Tris-HCl buffer (pH 9.0) to obtain OD 1.0 at 630 nm. The activity of purified enzyme was assayed under the assay conditions described, and then absorbance at 630 nm was measured. One unit (U) of PCL-degrading activity and hydrolysis of olive oil was defined as a 1 U/ml decrease in absorbance at 630nm under the assay conditions described by Nakamura *et al*. (2001). Esterase activity was assayed as described by Maeda *et al*. (2005), using five types of *p*-nitrophenyl (*p*NP) esters: *p*NP-acetate (C2), *p*NP-butyrate (C4), *p*NP-decanoate (C10), *p*NP-palmitate (C16) and *p*NP-stearate (C18). Fifty mM of *N*-succinyl-L-alanyl-L-alanyl-L-alanine 4-nitroanilide (Suc-$(Ala)_3$-*p*NA) was dissolved in 100 mM Tris-HCl buffer (pH 9.0). The enzyme reaction was incubated at 60 ^{o}C for 10min. The amount of *p*-nitroaniline (*p*NA) released was measured by the increase in absorbance at 410 nm. The caseinolytic activity was determined as described by Hagihara *et al*. (1958) and Li *et al.* (2008). Gelatinolytic activity assay was measured as described by Sukkhum *et al.* (2009b).

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5.2.4 Effects of inhibitors, reagents, and metal ions on purified enzyme activity

The purified enzyme was studied for the effects of different inhibitors (1, 5 and 10mM), consisting of phenylmethylsulfonylfluoride (PMSF), 1,4-dithiothreitol (DTT), ethylenediaminetetraacetate (EDTA), ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N′*,*N′*-tetraacetic acid (EGTA), and 1,10-phenanthroline hydrate (1,10-phen). Reagents such as 0.1, 0.2 and 0.5 % w/v of cetyl trimethyl ammonium bromide (CTAB), sodium dodecyl sulfate (SDS), Tween 20, Tween 80, Triton X-100, and 1, 5 and 10 mM of urea and β-mercaptoethanol (β-ME), were tested. The different metal ions used (1 and 4 mM) were K^{+}, Na^{+}, Ca^{2+}, Cu^{2+},Co^{2+}, Fe^{2+}, Mg^{2+}, $Mn^{2+}$and Zn^{2+}. The purified enzyme solution was co-incubated with different concentrations of each type of detergent, inhibitor and metal ion at 60 ^{o}C for 30 min; the residual enzyme activity was then measured using the standard PLLA-degrading enzyme assay. To study the effect of metal ions on enzyme activity, the purified enzyme was pre-treated with a final concentration of 5 mM EDTA for 24 h; the treated enzyme was then further dialyzed.

5.2.5 N-terminal amino acid sequences

The purified enzyme was subjected to determination of N-terminal amino acid sequence by SDS-PAGE as assayed by Alta Bioscience (Alta Bioscience Ltd., Birmingham, England) (Appendix B3).

6. Analysis

6.1 PLLA-degrading enzyme assay

PLLA-degrading enzyme activity was determined by measuring the decrease in the turbidity of emulsified PLLA, following the method of Sukkhum et al. (2009b). Emulsified PLLA as the substrate was prepared by dissolving 0.1 g PLLA in 4 mL dichloromethane. A PLLA suspension was sonicated in 100 mL of 100 mM Tris-HCl buffer (pH 9.0) (Sonics & Materials, Newtown CT, USA). The emulsion

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was diluted with 100 mM Tris-HCl buffer (pH 9.0) to obtain an optical density (OD) of 1.0 at a wavelength of 630 nm. PLLA-degrading enzyme activity was assayed by adding 0.25 mL of diluted enzyme solution and 2.25 mL of 0.1% emulsified PLLA; the mixture was then incubated at 60 ^{o}C for 30 min. One unit of PLLA-degrading activity was defined as a 1 unit decrease in optical density at a wavelength of 630 nm per min under the assay conditions described.

6.2 Growth determination

The growth of SSF and SmF was estimated using drop plate technique.

6.3 Protein analysis

Protein concentration was assayed using the Lowry method (Lowry *et al*., 1951), with bovine serum albumin as a standard.

6.4 Molecular weight estimation

The molecular weight of purified PLLA-degrading enzyme was determined by gel filtration chromatography and SDS-PAGE. The standard marker LMW (low molecular weight) Gel Filtration Calibration Kit was eluted through a column (1.5 × 35 cm) packed with Sephacryl S-100 gel filtration media (GE Healthcare Bio-Sciences, Uppsala, Sweden). The gel filtration column was eluted with 0.1 M Tris-HCl buffer (pH 9.0) at a flow rate of 0.6 ml/min. A calibration curve was constructed by plotting the gel phase distribution coefficient (K_{av}) versus the logarithm of the molecular weight (M_r). The molecular weight of purified PLLA-degrading enzyme was determined from the calibration curve. SDS-PAGE was studied using unstrained protein molecular weight markers (Fermentas, St. Leon-Rot, Germaney) as a standard.

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RESULTS AND DISCUSSIONS

1. Selection of PLA-degrading thermophilic filamentous bacteria

Eleven strains elucidated for their abilities to form a clear zone on an emulsified agar plate were secondarily selected and determined for their ability to produce PLLA-degrading enzyme. Among the 11 isolates, strain LP175, exhibited the highest PLLA-degrading activity, 5.07 U/ml, when the culture was grown at 50 ^{o}C for 4 days (Table 14 and Figure 12).

2. Identification of the selected PLLA-degrading producing strain

Among the 11 isolates, 16S rRNA gene sequences of strain LP175 were constructed phylogenetic tree as shown in Figure 13. Strain LP175 was closely related to the family *Thermoactinomycetaceae*. Strains LP175 was extremely closely related to *L. sacchari* DSM 43356^{T}, with sequence similarity of 99.9 %. Furthermore, the morphological and physiological characteristics of strain LP175 were similar to *L.sacchari* comb. (Lacey, 1971), as shown in Table 15. Strain LP175 was a Gram-positive, aerobic, thermophilic, filamentous and spore-forming bacterium. A white aerial mycelium and yellow-brown substrate mycelium were produced, but were unable to penetrate into the agar plate. The colony pattern of strain LP175 was a circular form and concentrically ringed, while *L. sacchari* comb. (Lacey, 1971) formed ridges on the agar surface. Spores formed singly were found on the aerial mycelium. The spores were globose under a light microscope, but they appeared angular under a scanning electron microscope because of a pattern of ridges on the spore surface (Figure 14). In regard to the physiological characteristics of strain LP175, the growth temperature ranges and optimum growth temperatures were 35–60 ^{o}C and 50–55 ^{o}C, respectively. Both *L. sacchari* LP175 and *L. sacchari* comb. could degrade casein, gelatin, soluble starch and Tween 80. Chitin, arbutin, esculin and tyrosine were degraded by *L. sacchari* LP175, while *L. sacchari* comb. degraded them to varying extents. Hypoxanthine was degraded only by *L. sacchari* LP175 but not by *L. sacchari* comb.. Furthermore, *L. sacchari* LP175 could degrade PLLA, while

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PLLA degradation of *L. sacchari* comb. was not determined. However, Sukkhum *et al.* (2009b) was previous reported that *L. sacchari* strain T11-7 was able to produce PLLA-degrading enzyme. *L. sacchari* LP175 showed substantial growth on NA with added cellulose, inositol, raffinose, rhamnose and sucrose; however, no growth was on these carbon sources evident for *L. sacchari* comb..

Table 14 Sampling locations for isolation of PLLA-degrading bacteria and their activity in basal medium containing 0.1% PLLA film at 50°C for 4 days

Sampling location	Isolates	Enzyme activity (U/ml)
Central Thailand		
Chaiyaphum province	C77	0.04±0.003
	C79	0.05±0003
	C86	0.03±0.025
	C88	0.04±0.010
	C89	0.08±0.007
Western Thailand		
Kanchanaburi province	K13	0.22±0.002
	K23	0.23±0.016
	K25	0.03±0.004
	KT176	0.07±0.033
Phetchaburi province	P43	0.03±0.001
Northern Thailand		
Lampang province	LP175	5.07±0.250

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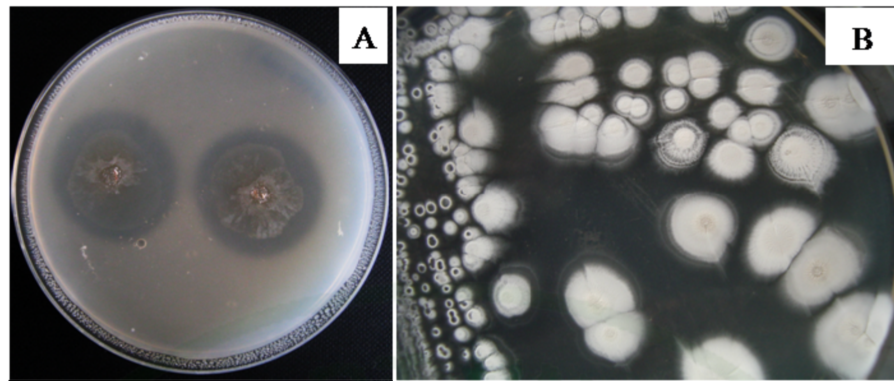

Figure 12 The formation of clear zone on emulsified-PLLA plate (A) and of colonies grown on Nutrient agar (B) from *L. sacchari* LP175

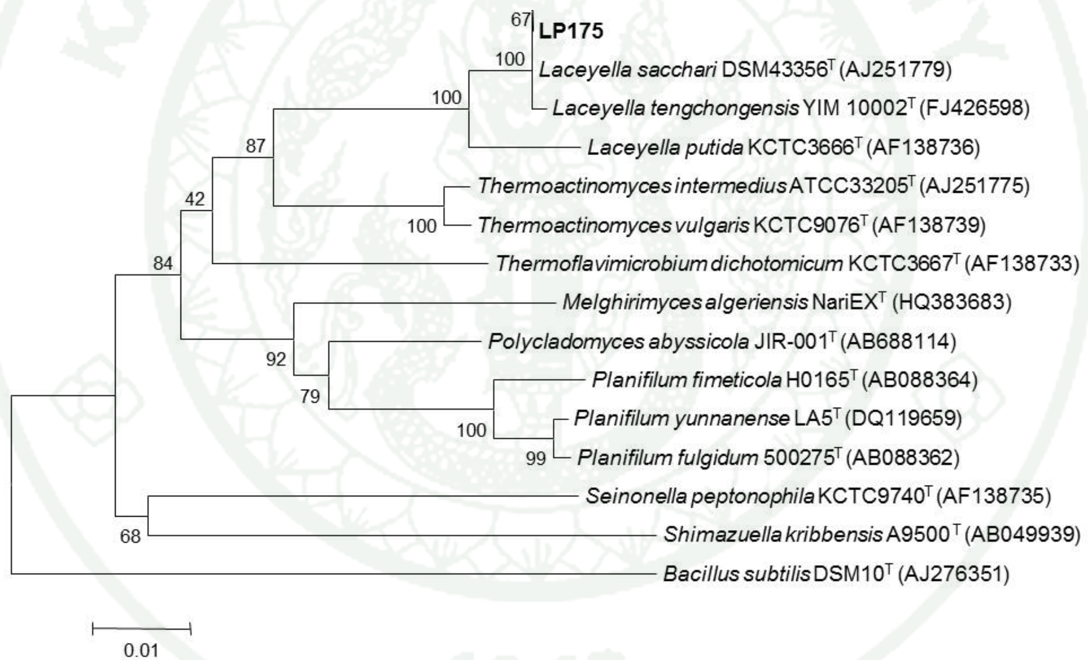

Figure 13 A neighbor-joining tree based on 16S rRNA sequences showing the phylogenetic relationships of strain LP175

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Table 15 Comparative phenotypic characteristics between *L. sacchari* LP175 and *L. sacchari* comb. (Lacey, 1971; Yoon et al., 2005)

Characteristics	*L. sacchari* LP175	*L. sacchari* comb.
Gram strain	+	+
Spores	+	+
Growth	Good	Poor
Aerial mycelium colour	White	White
Substrate mycelium	Yellow-brown	Yellow-brown
Lysis of aerial mycelium	Rapid within 3 days	Rapid, within 3 days
Mycelial septate	+	+
Colony morphologies:		
Colony surface	Concentrically ringed	Ridged
Growth conditions:		
Temperature range	35-60	35-65
Optimum temperature	50-55	55-60
Degradation of:		
Arbutin, Chitin, Esculin, Tyrosine	+	V
Cellulose	-	V
Casein, Gelatin, Soluble starch, Tween 80	+	+
Hypoxanthine	+	-
Growth in NaCl:		
1%	+	V
2-5%	-	-
Carbon utilization [a] :		
Glucose, Fructose	++	++
Mannitol	+++	++
Cellulose, Inositol, Raffinose, Rhamnose, Sucrose	+++	-

+, positive; -,negative; V, variable

[a] Carbon sources were added in NA to 1 % (w/v); -, no growth; +, growth similar to that on no carbon source medium; ++, growth similar to that on glucose added medium; +++, more growth than on glucose added medium.

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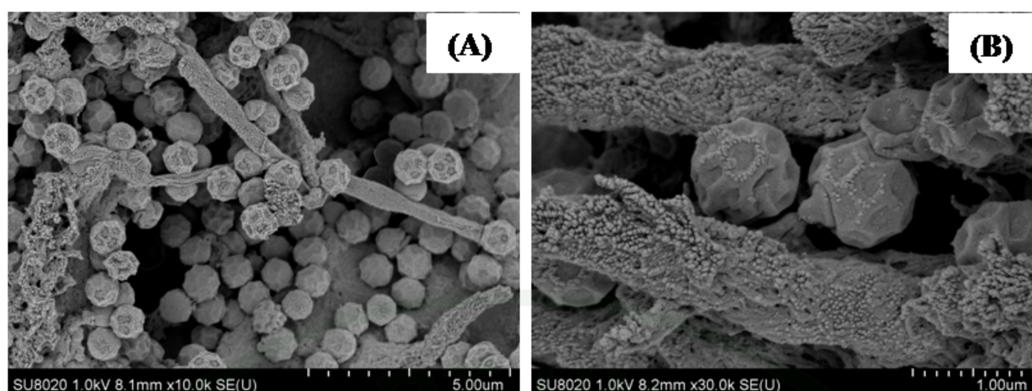

Figure 14 Scanning electron microscope of aerial mycelium spores in *L. sacchari* LP175 grown on NA for 1 week at 50 °C (A) Single spore formation, X 10,000 (B) Spore surface, X 30,000

3. Factor affecting for PLLA-degrading enzyme production in production medium by *L. sacchari* LP175

3.1 Effects of carbon sources on PLLA-degrading enzyme production

The effects of different proteinaceous substances and carbon sources on PLLA-degrading enzyme were evaluated by conventional one factor at a time (OFAT) in basal medium (Sukkhum *et al*., 2009a). Carbon was added to the production medium, with a total amount of 6 % of each carbon source. The best carbon source for enzyme production was cassava chip (13.00 U/ml) (Figure 15). Whilst adding sugars to the medium (glucose, xylose lactose mannose, raffinose and sucrose) had the effect of a decrease in PLLA-degrading activity. These results were similar to previous reports that glucose and lactose repressed protease produced by *Geobacillus caldoxylosilyticus* (Chen *et al*., 2004). Glucose completely repressed protease production, while proteins and peptides are necessary for protease production (Elibol and Moreira, 2005; El-Hadj-Ali *et al*., 2007; Akcan and Uyar, 2010). However, some micro-organisms found that sugars are the best carbon source for protease synthesis e.g. *Geobacillus caldoxylosilyticus* enhanced by sucrose (Chen *et al*., 2004), *B. licheniformis* N-2 enhanced by glucose (Nadeem *et al*., 2008), *Bacillus subtilis* PSKK96 enhanced by arabinose (Akcan and Uyar, 2010), *B. subtilis* 168

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enhanced by maltose (Vanitha *et al*., 2014). These results indicate that the addition of sugars to the medium was not sufficient to promote the PLLA-degrading enzyme production by *L. sacchari* LP175.

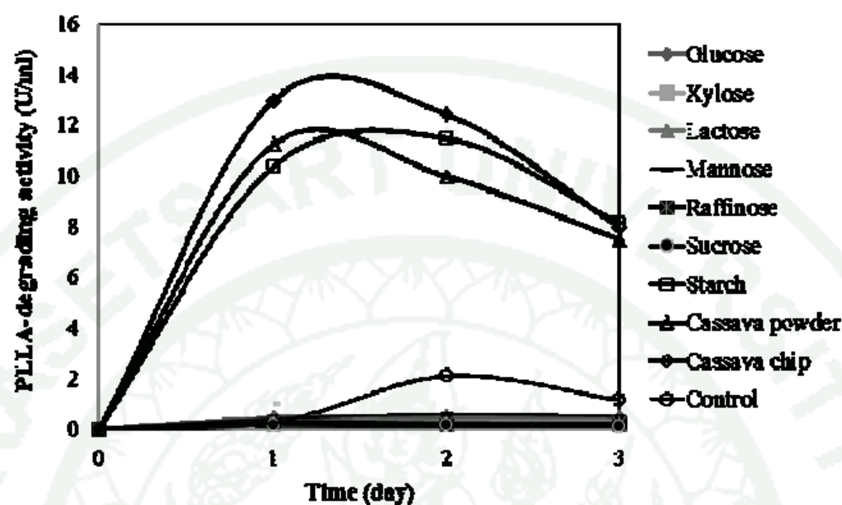

Figure 15 Effects of different carbon sources on PLLA-degrading enzyme production by *L. sacchari* LP175

3.2 Effects of proteinaceous substances on PLLA-degrading enzyme production

The effects of various proteinaceous substances such as silk powder, sericin powder, casein, casamino acids, tryptone, peptone, gelatin, yeast extract and soybean meal on PLLA-degrading enzymes were studied. The total amount of nitrogen was added to the production medium with 3.68 % of each proteinaceous substances. As shown in Figure 16, soybean meal and gelatin (3.67 %) gave the maximum PLLA-degrading enzyme activity which was 3.47 and 1.08 U/mL from *L. sacchari* strain LP175. In similar previous reports, soybean meal (1% w/v) has been used as the best inducer for alkaline protease production, e.g. *Teredinobacter tunirae* (Elibol and Moreira, 2005), *B. licheniformis* N-2 (Nadeem *et al*., 2008).

Many proteins such as silk fibroin and elastin, and some peptides and amino acids can be stimulated to produce PLLA-degrading enzyme from micro-organisms

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(Tokiwa *et al*., 2009). The nitrogen sources used as inducers for the PLLA-degrading enzyme production are low yeast extract for *Amycolatopsis* sp. strain 41 (Ikura and Kudo, 1999); silk powder for Amycolatosis sp. strain 41 (Pranamuda *et al*., 2001); gelatin for yeast *Tritirachium album* ATCC 22563 (Jarerat and Tokiwa, 2001), *Saccharothrix waywayandensis* (Jarerat and Tokiwa, 2003), *Kibdelosporangium aridium* (Jarerat *et al*., 2003) and *Actinomodura keratinolytica* T16-1 (Sukkhum *et al*., 2009b). Although the type of nitrogen source for PLLA-degrading enzyme production from *L. sacchari* strain LP175 was different from the previous reports, it has close similarity in nutrient components.

Generally, the protease production required amino acids and short peptides as organic proteinaceous substances for enhancing the enzyme activity (Gomaa, 2013). Soybean meal has a high content of protein (44% to 49%) and is rich in amino acids (lysine, tryptophan, threonine, isoleucine and valine) (Baize, 2000). On the other hand, soybean meal enhanced alkaline protease production by wild and EMS induced mutant strains of *Bacillus subtilis* IH-72EMS8 (Mukhatar and Haq, 2013) and *Bacillus* sp. (Saurabh *et al*., 2007). These results suggested that the proteins from soybean meal can promote the PLLA-degrading enzyme production of *L. sacchari* LP175 which differs from the previous reports. Many previous reports found that soybean meal was the best inducer and a source of both carbon and nitrogen for protease production from *Conidiobolus coronatus* and *Bacillus* sp. (SBP-29) (Saurabh et al., 2007). Both cassava and soybean meal are inexpensive substrates. Therefore, cassava chip and soybean meal were used as carbon and nitrogen sources, respectively, for further study.

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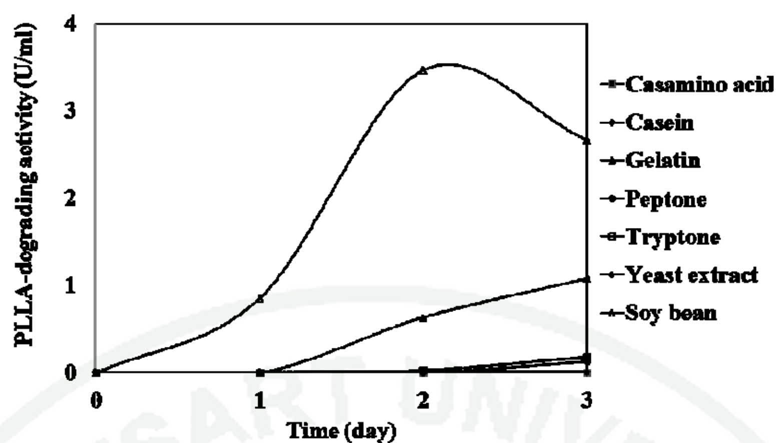

Figure 16 Effects of different proteinaceous substances on PLLA-degrading enzyme production by *L. sacchari* LP175

4. Optimization of fermentation process for PLLA-degrading enzyme production from *L. sacchari* LP175 using agricultural product as substrates

4.1 Comparison of PLLA-degrading enzyme production in solid state fermentation (SSF) and submerged fermentation (SmF)

The *L. sacchri* LP175 can be grown on some agricultural products such as wheat bran, cassava chip and soybean meal, which is cheapest substrates for culturing microorganisms. In this work we focused on the production of PLLA-degrading enzyme by *L. sacchri* LP175 using low cost substrates. Furthermore, these substrates are suitable enhanced protease production.

4.1.1 Solid state fermentation

The effect of single or mixed composition agricultural products (wheat bran, cassava chip and soybean meal) used as substrates for PLLA-degrading enzyme production are shown in Figure 17. The Table 16 shows the interactions of three substrates on PLLA-degrading enzyme in seven experiments. The PLLA-degrading activity in the medium that contained only soybean meal and cassava chip was 1.2 and 1.3 U/g, respectively. However, when the medium was composed of

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soybean meal (1/2) and cassava chip (1/2) the results showed a two-fold increase in enzyme activity

Table 16 The experimental mixtures of agricultural products for PLLA-degrading enzyme activity at 4 days cultivation by *L. sacchari* LP175

Experiment	Independent variables			PLLA-degrading activity (U/g)
	Wheat bran (X1)	Soybean meal (X2)	Cassava chip (X3)	
1	1	0	0	1.0
2	0	1	0	1.2
3	0	0	1	1.3
4	1/2	½	0	0.74
5	1/2	0	1/2	0.95
6	0	½	1/2	2.74
7	1/3	1/3	1/3	1.02

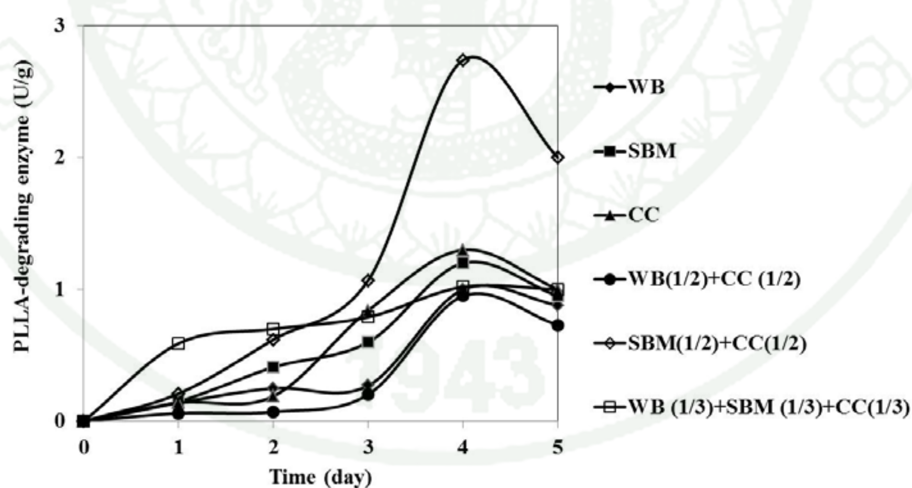

Figure 17 Effect of agricultural products for PLLA-degrading enzyme production during 4 days by *L. sacchari* LP175

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4.1.2 Submerged fermentation

In submerged fermentation studies (SmF), the culture was studied on the basal medium consisting of (g/l) 10% cassava chip; 10 % soybean meal and 0.035 % PLLA-powder. The production of PLLA-degrading enzyme by *L. sacchari* LP175 was 5.18 U/ml as shown in Table 17.

PLLA-degrading enzyme production by *L. sacchari* LP175 in SmF and SSF using soybean meal and cassava chip as nitrogen and carbon sources were investigated. The enzyme activity value obtained in SSF was lower than that from SmF (Table 17). Furthermore, the time for enzyme production in SmF was faster than in SmF. The soybean meal and cassava chip were both inducers and nutrients for the PLLA-degrading enzyme produced. In addition, the dissolved oxygen in the medium during cultivation is important for PLLA-degrading enzyme by the strain. Azeredo et al. (2006) found that the protease production from *Streptomyces* sp. 594 in SSF exhibited lower than in SmF using a keratinous waste. The increase in pH during the cultivation of keratin hydrolysis is important, which involves deamination reactions and the keratinolytic potential of micro-organisms. Therefore, the optimization of PLLA-degrading enzyme production was investigated in SmF.

Table 17 Comparison of PLLA-degrading enzyme production under solid sate and submerged fermentation by *L. sacchari* LP175

Bacteria	PLLA-degrading enzyme production	
	SSF (U/g)	SmF (U/ml)
L. sacchari LP175	2.74 (4 days)	5.18 (3days)

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4. Optimization of fermentation process for PLLA-degrading enzyme production from *L. sacchari* LP175 using agricultural products as substrates

4.1 Effects of basal medium components on PLLA-degrading enzyme production

Different medium formoulas obtained the addition of each mineral salts and their composition to the suspension of cassava chip and soybean maeal were tested for PLLA-degrading enzyme production by LP175. The maximum activity (55.87 U/ml) was obtained under the medium formula 4, which consisted of (g/l): 2 g K_2HPO_4, 1 g KH_2PO_4, and 0.2 g $MgSO_4.7H_2O$, but when ammonium sulfate was added to the medium, the enzyme activity dropped two-fold (Figure 18). The enzyme activity was low value in the medium consisting of $(NH_4)_2SO_4$.

The results indicated that the production was stimulated by phosphate and Mg^{+2}. Phosphate has an important role to play in enzyme reactions of primary metabolism, DNA, RNA and protein synthesis, carbohydrate metabolism, cellular respiration control and ATP levels (Gomaa, 2013). An important role of Mg^{+2} was to increase and stabilize enzyme production (Paliwal *et al*., 1994). However, the enzyme production was inhibited by ammonium sulfate. Similar finding had been reported by Saurabh *et al.* (2007), who observed that ammonium ion was a strong catabolic repression for protease production. This result was not similar to the result reported by Sukkhum *et al.* (2009b), who reported that PLLA-degrading enzyme production is not repressed by ammonium sulfate.

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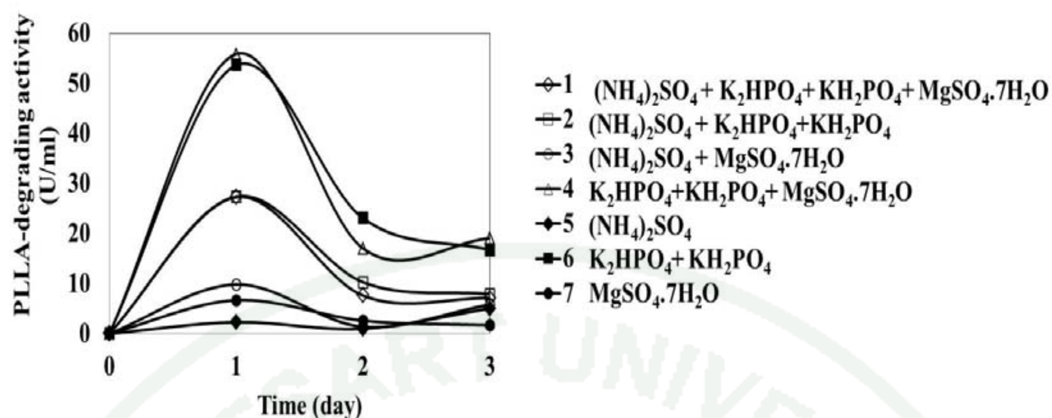

Figure 18 Effects of different basal medium components on PLLA-degrading enzyme production by *L. sacchari* LP175

4.2 Effect of initial pH on PLLA-degrading enzyme production

The effect of initial pH was measured in production medium, which consisted of (g/l): 2 g K_2HPO_4, 1 g KH_2PO_4, and 0.2 g $MgSO_4.7H_2O$ by adding cassava chip as a nitrogen source, soybean meal as a carbon source and PLLA powder as an inducer. The initial pH for maximum production of PLLA-degrading enzyme by *L. sacchari* LP175 was 7.0 (57.6 U/ml) as shown in Figure 19.

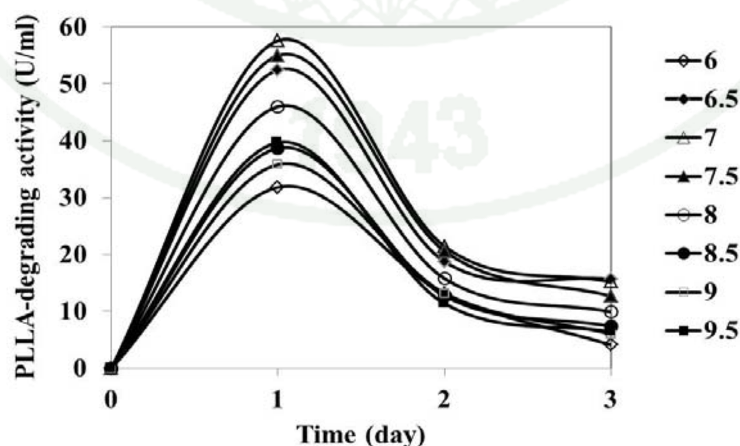

Figure 19 Effect of initial pH on PLLA-degrading enzyme production by *L. sacchari* LP175

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4.3 Factors affecting PLLA-degrading enzyme by statistical design

4.3.1 Regression model of response

On the basis of results obtained in the optimum medium for maximum PLLA-degrading enzyme production and growth, the results were compiled. The optimal medium contained (g/l): 2 g K_2HPO_4, 1 g KH_2PO_4, and 0.2 g $MgSO_4.7H_2O$, cassava chip, soybean meal and PLLA powder. However, only three key independent variables, namely cassava chip (X_1), soybean meal (X_2) and PLLA powder (X_3), were designed to obtain the best conditions for the maximum PLLA-degrading enzyme production by a CCD of RSM. A total of 17 runs performed using a CCD contained three parts: full factorial design (2^3, runs 1 to 8), star points or axial points (3X2, runs 9 to 14) and central points repeated (runs 15 to 17). The experimental and predicted values of enzyme activities are shown in Table 18.

Table 18 Experimental design used in response surface methodology of three independent variables, cassava chip (X_1), soybean meal (X_2) and PLLA powder (X_3) with three centre points, and the observed and predicted PLLA-degrading activity

Run No.	Level			Actual level			PLLA-degrading activity (U/ml)		Growth (log CFU/ml)	
	X_1	X_2	X_3	X_1	X_2	X_3	Observed	Predicted	Observed	Predicted
1	-1	-1	-1	3.0	1.0	0.15	30.63	32.86	7.34	7.18
2	-1	-1	1	3.0	1.0	0.45	28.27	34.26	7.32	7.28
3	-1	1	-1	3.0	2.0	0.15	32.03	31.61	7.45	7.34
4	-1	1	1	3.0	2.0	0.45	44.07	40.30	6.90	6.67
5	1	-1	-1	6.0	1.0	0.15	43.07	44.59	7.31	7.19
6	1	-1	1	6.0	1.0	0.45	46.73	44.90	8.08	7.85
7	1	1	-1	6.0	2.0	0.15	54.20	45.95	7.36	7.05
8	1	1	1	6.0	2.0	0.45	58.02	53.54	7.11	6.93
9	-1.68	0	0	2.25	1.5	0.3	44.23	40.10	7.26	7.44
10	1.68	0	0	7.02	1.5	0.3	48.83	55.31	7.36	7.70

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Table 20 (Continued)

Run No.	Level			Actual level			PLLA-degrading activity (U/ml)		Growth (log CFU/ml)	
	X_1	X_2	X_3	X_1	X_2	X_3	Observed	Predicted	Observed	Predicted
11	0	-1.68	0	4.5	0.66	0.3	34.75	28.95	6.65	6.81
12	0	1.68	0	4.5	2.34	0.3	26.17	35.16	5.85	6.18
13	0	0	-1.68	4.5	1.5	0.05	45.50	47.37	7.26	7.52
14	0	0	1.68	4.5	1.5	0.55	53.50	54.87	7.26	7.50
15	0	0	0	4.5	1.5	0.3	68.25	67.68	7.28	7.39
16	0	0	0	4.5	1.5	0.3	66.65	67.68	7.53	7.39
17	0	0	0	4.5	1.5	0.3	67.92	67.68	7.49	7.39

The second order polynomial equation 4 and 5 were used to predict the value of the enzyme production as follows:

$$Y_1 = -156.247 + 35.608 X_1 + 143.959 X_2 + 143.050 X_3 + 0.870 X_1X_2 - 1.222 X_1X_3 + 24.267 X_2X_3 - 3.598 X_1^2 - 50.487 X_2^2 - 264.922 X_3^2 \quad (4)$$

where Y_1 is the predicted response (PLLA-degrading enzyme production); X_1, X_2 and X_3 are coded values of cassava chip, soybean meal and PLLA powder, respectively.

$$Y_2 = 4.679 - 0.265 X_1 + 4.657 X_2 - 0.024 X_3 - 0.102 X_1X_2 + 0.606 X_1X_3 - 2.583 X_2X_3 + 0.031 X_1^2 - 1.267 X_2^2 + 1.916 X_3^2 \quad (5)$$

where Y_2 is the predicted response (growth); X_1, X_2 and X_3 are coded values of cassava chip, soybean meal and PLLA powder, respectively.

The corresponding analysis of varience (ANOVA) of PLLA-degrading enzyme production and growth are presented in Table 19 and 20, respectively. The statistical significance of the regression model was checked by Fisher's-test value (F-test) and the analysis of variance (ANOVA) explained by the

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model was given by the multiple coefficient of determination, R squared (R^2) value. The $R1^2$ and $R2^2$ is 0.88 and 0.78, respectively, implie that the samples variation of 88.8% and 78.0% are attributed to the independent variables on PLLA-degrading enzyme production and growth, respectively. The R^2 value also indicates that only 11.2% of the variation cannot be explained by the model on on PLLA-degrading enzyme production, while the growth had only 22.0%. Normally, a regression model having an R^2 value higher than 0.9 is considered to have a very high correlation. The closer the value of R (correlation coefficient) to 1, the better the correlation between the experimental and predicted values. Here, the value of R (0.931) indicated a close agreement between the experimental results and the theoretical values predicted by the model equation. Thus, the fit of the model was statistically significant at the 95%

Table 19 Analysis of variance (ANOVA) for the model regression representing PLLA-degrading enzyme activity

Effect	Sums of squares	Df	Mean square	F	*p*-value
Regress.	2623.25	9	291.47	6.19	0.013
Residual	329.58	7	47.08		
Total	2952.83				

df, degree of freedom; significant level, 95%; $R1^2$, 0.888

Table 20 Analysis of variance (ANOVA) for the model regression representing growth

Effect	Sums of squares	Df	Mean square	F	*p*-value
Regress.	2.62	9	0.29	2.74	0.09
Residual	0.74	7	0.11		
Total	3.36				

$R2^2$ = 0.78; df, degrees of freedom; significance level = 95%

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To assess the significance of each term (three linear coefficients, X_1, X_2 and X_3; three cross product term, X_1X_2, X_1X_3 and X_2X_3 ; three quadratic term, X_1^2 , X_2^2 and X_3^2 on the PLLA-degrading activity and growth was determined by a student's t-test and p value as shown in Table 21 and 22,respectively. In this study, the p-values less than 0.05 are considered significant effect on the response. The parameter estimates and the corresponding p-values suggest that, among the independent variables used in the study, X_1 (cassava chip), X_2 (soybean meal) and X_3 (PLLA powder) had a significant effect on PLLA-degrading enzyme production. The quadic term, X_1, X_2 X_1^2, X_2^2 and X_3^2 also had a significant effect. The other models terms were not found to be significant. These results indicated that the interactions between the two variables were not found to contribute to the response at a significant level. The term X_2 and X_2^2 showed significant influence on the both PLLA-degrading enzyme production and growth.

Table 21 Result of regression analysis of the central composite design for PLLA-degrading enzyme activity

Term	Coefficient	T-statistic	p-value
Intercept	44.083	-3.544	0.009
X_1	10.768	3.307	0.013 [a]
X_2	30.209	4.765	0.0020[a]
X_3	88.706	1.613	0.1509
X_1X_2	3.2346	0.2690	0.7957
X_1X_3	10.7821	-0.1133	0.9129
X_2X_3	32.3462	0.7502	0.4776
X_1^2	0.9814	-3.6665	0.008[a]
X_2^2	8.1154	-6.2211	0.000[a]
X_3^2	91.3793	-2.8992	0.023 [a]

[a] Statistically significant at 95% of confidence level

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Table 22 Result of regression analysis of the central composite design for growth

Term	Coefficient	T-statistic	p-value
Intercept	2.09	2.24	0.06
X_1	0.51	-0.52	0.62
X_2	1.43	3.25	0.01[a]
X_3	4.21	-0.01	0.99
X_1X_2	0.15	-0.66	0.53
X_1X_3	0.51	1.18	0.27
X_2X_3	1.53	-1.68	0.14
X_1^2	0.05	0.67	0.52
X_2^2	0.39	-3.29	0.01[a]
X_3^2	4.34	0.44	0.67

4.4.2 Localisation of optimum condition

The three dimensional (3D) response surface plots and the corresponding contour plot show the effects of cassava chip, soybean meal and PLLA powder concentrations on the PLLA-degrading enzyme production. Figure 20 A shows the effect of cassava chip and soybean meal while keeping the PLLA powder at zero level. The result indicated that the highest yield of the PLLA-degrading enzyme production were up to 0.445 - 0.605% (w/v) cassava chip and 0.145-0.135% (w/v) soybean meal, respectively. The PLLA-degrading enzyme production was studied to assess an interaction between cassava chip and PLLA powder by keeping the soybean meal at a central level (Figure 20 B). The optimum ranges of cassava chip and PLLA powder were 0.51-0.59% (w/v) and 0.031-0.043% (w/v), respectively. Figure 20 C shows that the maximum PLLA-degrading enzyme production occurred at soybean meal of 0.142-0.175% (w/v) and of 0.028-0.042% (w/v) PLLA powder, which is fixed at its central level. This suggested that the optimal ratio between cassava chip: soybean for maximum PLLA-degrading production was 3:1. The concentration of PLLA powder within the tested range did not affect the yield of PLLA-degrading

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enzyme. The optimum ranges of soybean meal and cassava chip concentrations for growth were 1.0-1.4 g/l and ion4-5 g/l, respectively (Figure 21).

4.4.3 Model adequacy checking

It is necessary to check the fitted model to ensure that it provides an adequate approximation to the real system. The model shows an adequate fit, proceeding from the investigation and optimization of the fitted response surface likely give poor or misleading results. The residuals from the least squares fit play an important role in judging model adequacy (Myers and Montgomery, 2002). By constructing a normal probability plot of the residuals, a check was made for the normality assumption, as given in Figure 22 A for PLLA-degrading enzyme production and Figure 22 B for growth. The normality assumption was satisfied as the residual plot approximated along a straight line. The plots were satisfactory, so it was concluded that the empirical model was adequate to describe the PLLA-degrading activity and growth by response surface.

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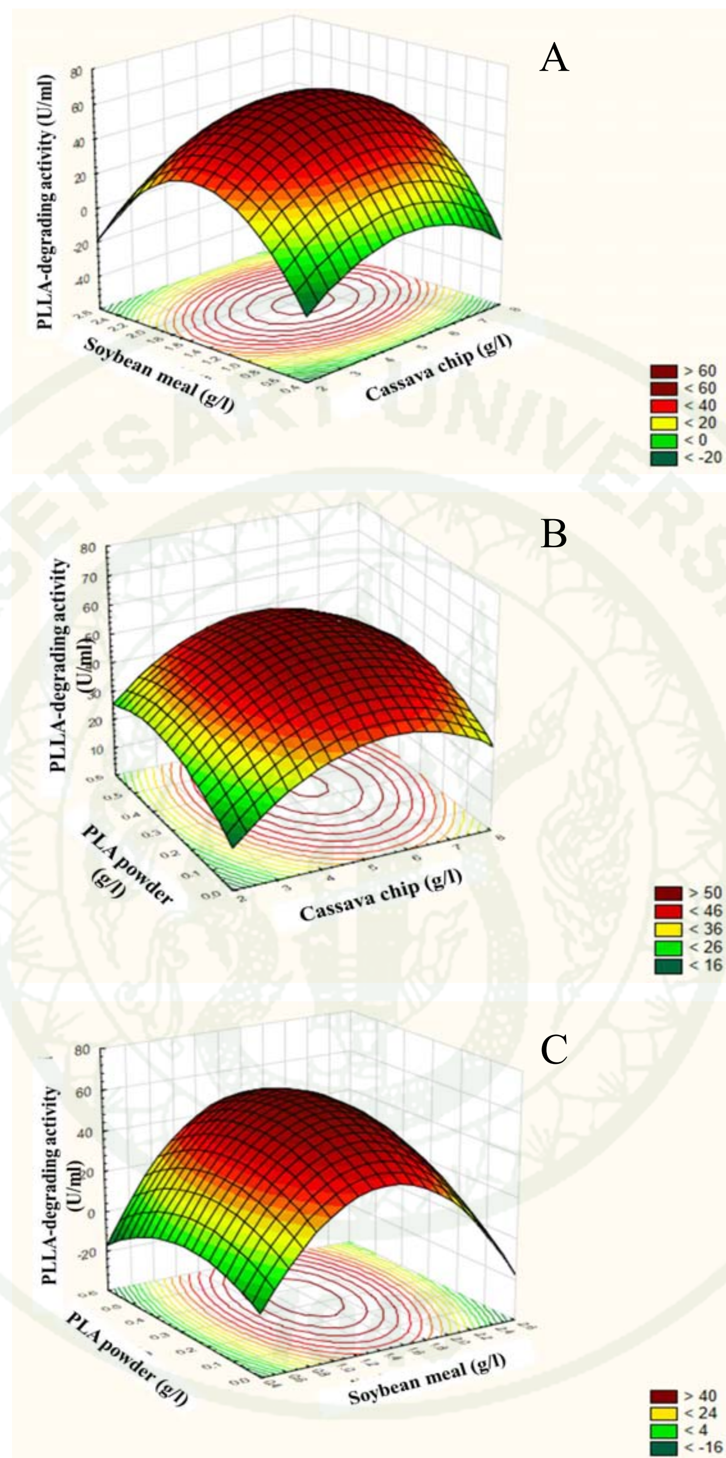

Figure 20 Response surface described by the model, representing PLLA-degrading enzyme activity (U/ml) as a function of cassava chip and soybean meal (A), cassava chip and PLLA powder (B) and soybean meal and PLLA powder concentrations (C)

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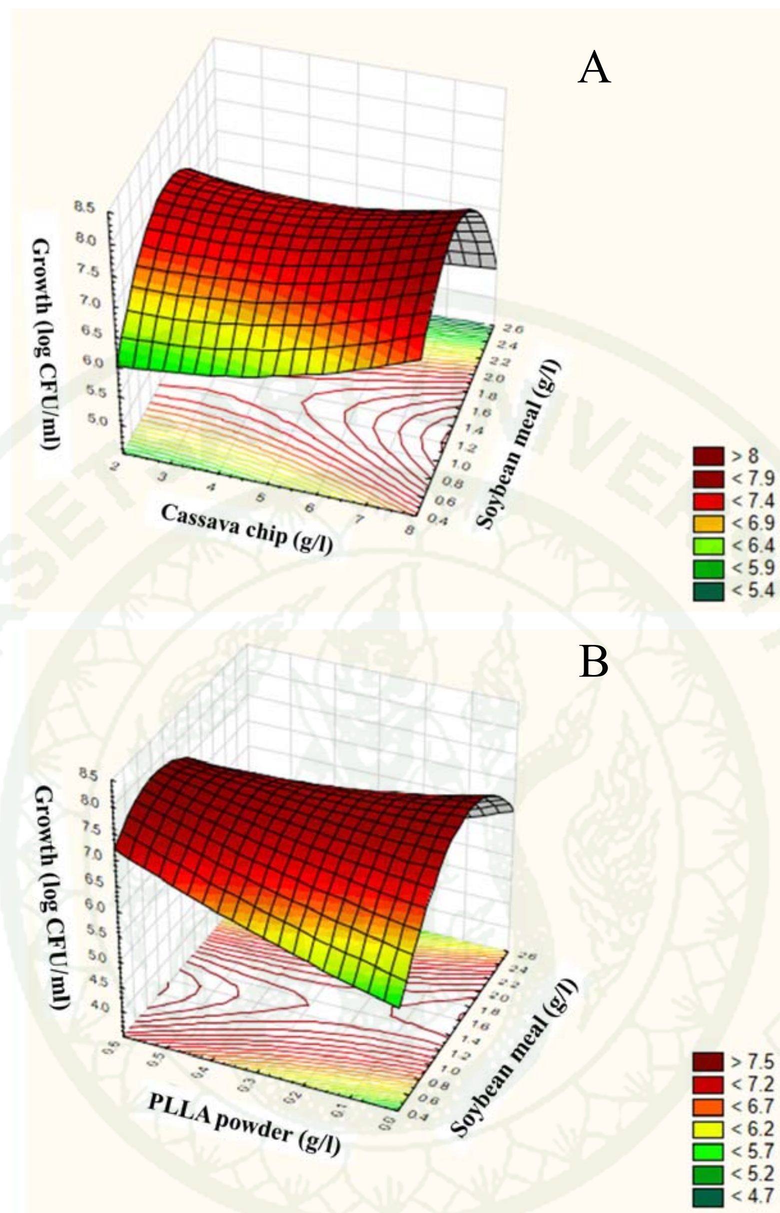

Figure 21 Response surface described by the model, representing growth (log CFU/ml) as a function of cassava chip and soybean meal (A) and soybean meal and PLLA powder concentrations (B)

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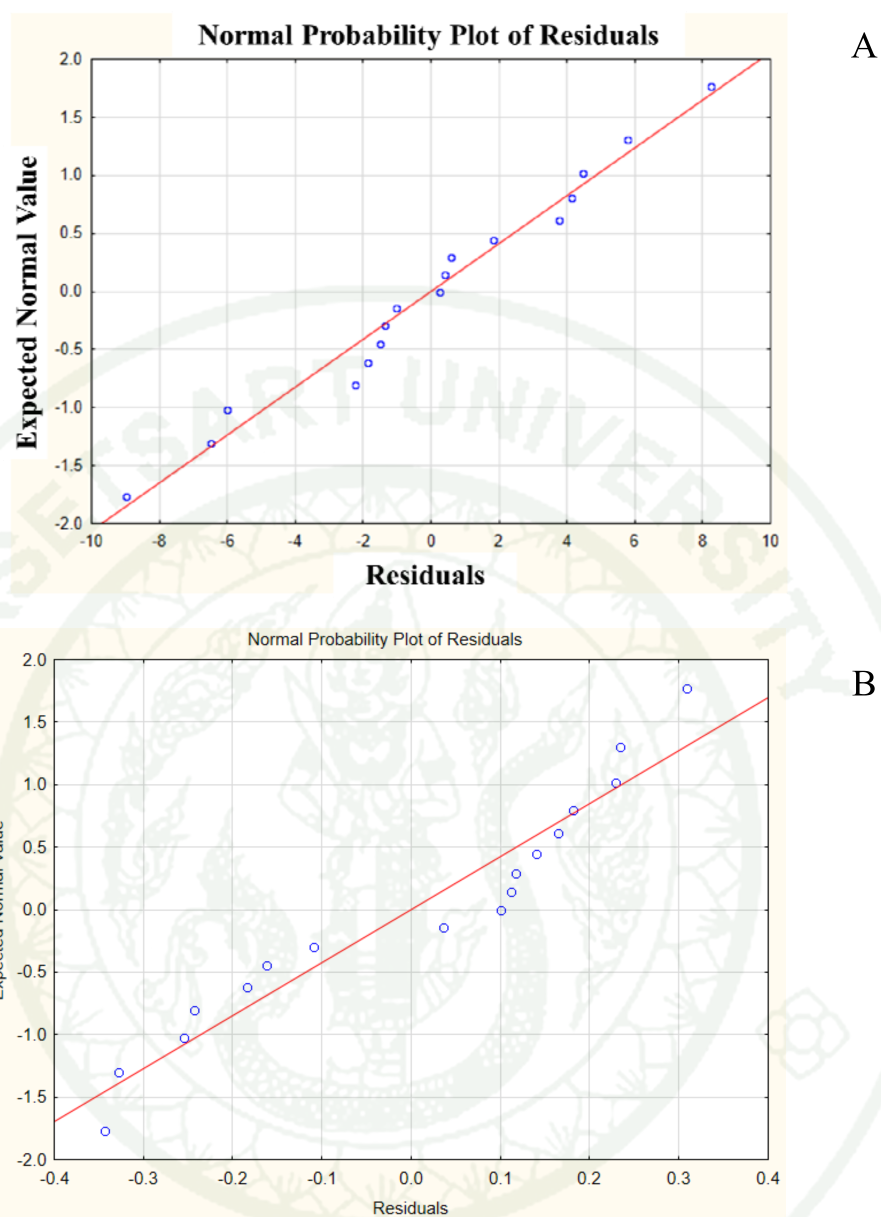

Figure 22 Normal probability plot of residuals for PLLA-degrading enzyme (A) and growth (B)

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4.4.4 Statictical model validation

To confirm the predicted response from the medium composition obtained, validation of the model was carried out under optimum conditions and the statistical model and regression equation was validated. The model was validated by canonical analysis of the response surface. The best optimized values of a maximum PLLA-degrading activity had the following critical values: 4.64 g/l of cassava chip; 1.53 g/l of soybean meal; 0.31g/l of PLLA powder. The closeness of the predicted PLLA-degrading activity for these conditions was 68.25 U/ml in shake flask (Figure 23). The maximum experimental response for PLLA-degrading enzyme production was 65.5 U/ml after 24 h cultivation with productivity of 2.73 U/ml/h. The enzyme productivity obtained from *L. sacchari* LP175 was 5.93 times higher than *Actinomadura keratinolytica* T16-1 (0.46 U/ml/h) (Sukkhum *et al*., 2009b).

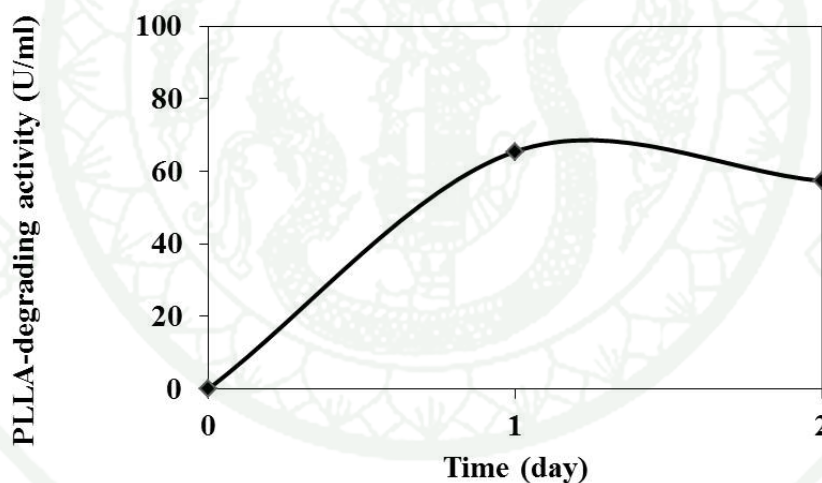

Figure 23 PLLA-degrading production by *L. sacchari* LP175 from optimized medium

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4.5 Physical factors affecting PLA-degrading enzyme production in an airlift fermenter

4.5.1 Effect of aeration rate on PLLA-degrading enzyme production

The optimized medium was used to study factors affecting production of PLLA-degrading enzyme by *L. sacchari* LP175 in a working volume of 2 L of airlift fermenter with an aeration rate of 0.25, 0.5 and 0.75 vvm and controlled pH 7.0 at 50°C. The maximum amount of PLLA-degrading enzyme was produced when the aeration rate was kept at 0.5 vvm (94.4 U/ml) (Figure 24). With the aeration rate at 0.25 and 0.75 vvm reduced enzyme activity was obtained at 70.23 and 42.93 U/ml, respectively. These results indicate that the enzyme productivity increased with an increase in the aeration rate up to 0.5vvm but decreased at a higher aeration rate (0.75 vvm). Rate of oxygen transfer plays an important role in overall microbial metabolism and different micro-organisms behave diversely in different conditions of oxygen supply (Singh *et al*., 2007).

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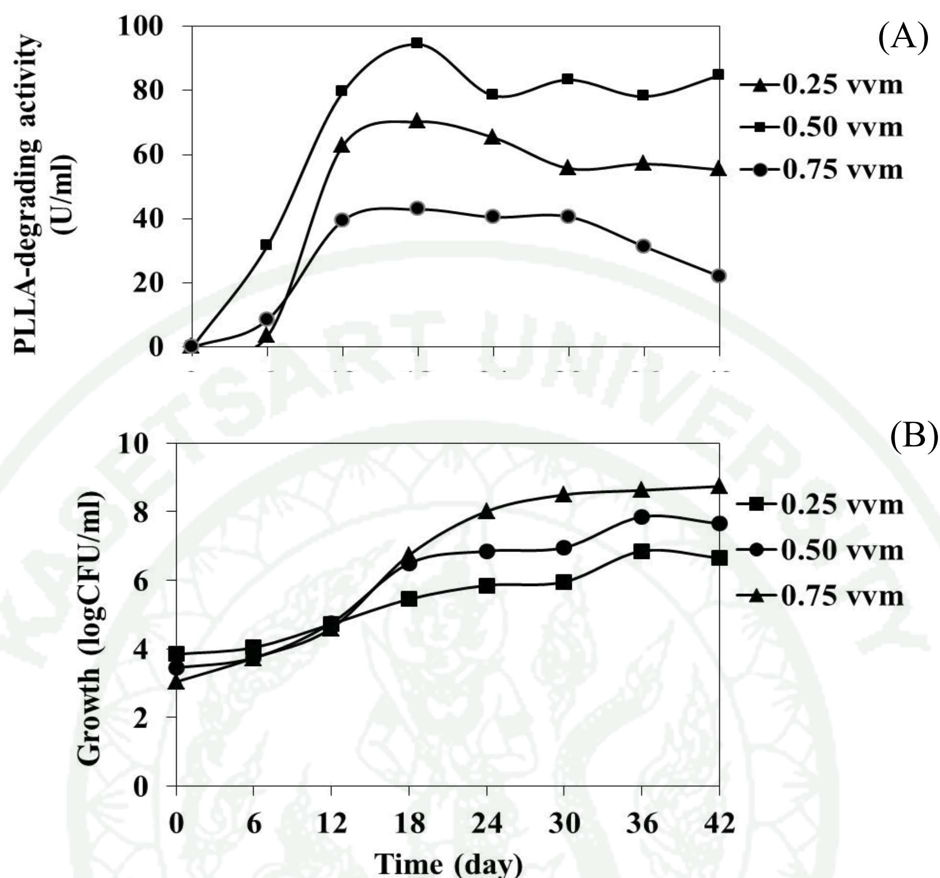

Figure 24 Effects of aeration rate (A) and growth (B) on PLLA-degrading enzyme production by *L. sacchari* LP175 in an airlift fermenter at 50 ˚C and pH 7.0

4.5.2 Effect of temperature on PLLA-degrading enzyme production

The optimized medium, pH 7.0 and aeration rate of 0.5 vvm were used to study on effect of temperatures for PLLA-degrading enzyme production by *L. sacchari* LP175. Figure 25 shows the PLLA-degrading enzyme production profile, demonstrating that the maximum PLLA-degrading activity was obtained when the strain was cultured at 40 °C for 24 h (68.74 U/ml) and 50°C for 18 (94.4 U/ml).

PLLA-degrading enzyme production by strain LP175 is summarised in Table 23. Casava chip and soybean meal was used as a carbon source and proteinaceous substance, respectively for un-optimized medium. The enzyme activity 57.6 U/ml was obtained. The concentrations of optimized medium by using CCD in

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shaking flasks and airlift fermentation were 4.64 g/l of cassava chip, 1.53 g/l of soybean meal and 0.31 g/l of PLLA powder. The statistical model was validated in an airlift fermenter using optimized medium under the conditions: aeration rate of 0.5 vvm, initial pH 7.0 and temperature 50 ˚C. The enzyme activity increased up to 94.4 U/ml under these conditions. Strain LP175 gave higher PLLA-degrading enzyme productivity (125.87 U/ml/h) than *A. keratinilytica* T16-1 (80 U/ml/h) in airlift fermentation (Sukkhum *et al*., 1999b). On the other hand, strain LP175 (18 h) produced a faster yield of PLLA-degrading enzyme than *Amycolatopsis orientalis* (72 h) (Jarerat *et al*., 2006) and *A. keratinilytica* T16-1 (72h) (Sukkhum *et al*., 1999b).

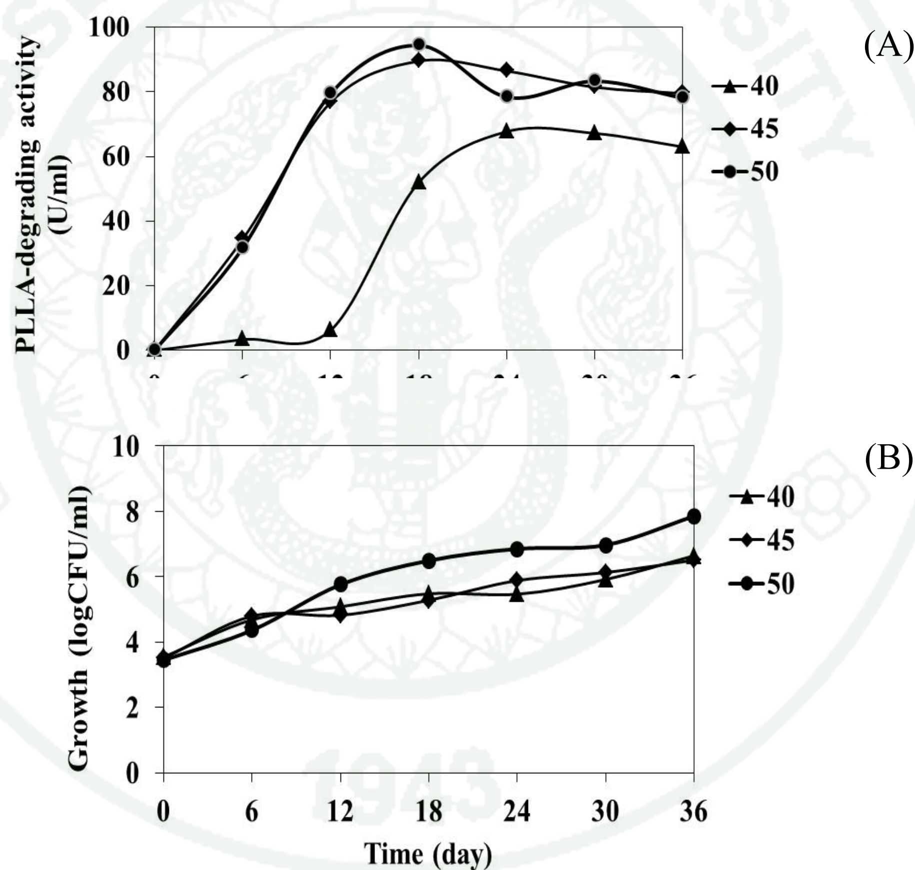

Figure 25 Effects of temperature (A) and growth (log CFU/ml) (B) on PLLA-degrading enzyme production by *L. sacchari* LP175 in an airlift fermenter at pH 7.0 and aeration rate of 0.5 vvm

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Table 23 PLLA-degrading enzyme production by *L. sacchari* LP175 using agricultural products

Medium	Conditions	PLLA-degrading enzyme production (U/mL)
Un-optimized medium	**Shaking fasking**	57.6
1.5 g/l cassava chip	Shaking speed 150 rpm	
2.64 g/l soybean meal	Temperature at 50 ^{0}C	
0.35 g/l PLLA powder	Initial pH at 7.0	
2.0 g/l K_2HPO_4		
1.0 g/l KH_2HPO_4		
0.2 g/l $MgSO_4.7H_2O$		
Optimized medium	**Shaking flask**	65.5
4.64 g/l cassava chip	Shaking speed 150 rpm	
1.53 g/l soybean meal	Temperature at 50 ^{0}C	
0.31 g/l PLLA powder	Initial pH at 7.0	
2.0 g/l K_2HPO_4	**2 L airlift fermentor**	94.4
1.0 g/l KH_2HPO_4	Aeration rate of 0.5 vvm	
0.2 g/l $MgSO_4.7H_2O$	Temperature at 50 ^{0}	
	Controlled pH at 7.0	

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5. Purification and characterization of PLLA-degrading enzyme

L. sacchari LP175 was cultured on production medium (Sukkhum *et al*. 2009a) for 2 days at 50 ^{o}C.

5.1 Purification of the PLLA-degrading enzymes

The PLLA-degrading enzyme with activity of 49.6 U/mL obtained from strain LP175 cultured on basal medium supplemented with 0.035 % (w/v) PLLA powder for 2 days was used for enzyme purification. The supernatant was changed by counterions to pH 5.0 by dialysis with 20mM acetate buffer (pH 5.0) before being applied to a CM Sepharose column. All active fractions were pooled, and the buffer was immediately changed to 100mM Tris-HCl buffer (pH 9.0) since the enzyme was not stable at pH 5.0. The summary of purification procedures of PLLA-degrading enzyme from strain LP175 is shown in Table 24. The purified enzyme was purified 15.3-fold with a yield of 48.1% and a specific activity of 328 U/mg of protein. In general, the purification of PLLA-degrading enzyme requiring multiple steps resulted in lower yield and purification fold (Li *et al*., 2008; Pranamuda *et al.,* 2001; Sakai *et al*., 2001; Sukkhum *et al*., 2009b).

Table 24 Purification steps of a PLLA-degrading enzyme produced by *L. sacchari* LP175

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture supernatant	300	6448	21.5	100	1
Dialyzed culture supernatant	178.2	4811	27.0	74.6	1.3
CM-Sepharose	9.5	3100	328	48.1	15.3

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5.2 Substrate specificity of purified PLLA degrading enzyme

The hydrolysis of various substrates of purified PLLA-degrading enzyme from *L. sacchari* LP175 (Table 25) indicated that PLLA had the highest substrate specificity (388 U mg/protein). Furthermore, the PLLA-degrading enzyme exhibited caseinolytic and gelatinolytic activities, with 340 and 262 U/mg of protein, respectively. The purified enzyme was weakly specific toward the peptide *p*-nitroanilide. However, lipase and esterase activities were not found. The majority of purified PLLA-degrading enzymes are able to degrade both peptide bonds and ester bonds, depending on the strain: e.g. serine protease from *Amycolatopsis* sp. strain K104 degrades casein and fibrinogen (Nakamura *et al*., 2001); purified enzyme from *Amycolatopsis* sp. strain 41 degrades casein, silk fibroin and Suc-$(Ala)_3$-*p*NA (Pranamuda *et al*., 2001); and multiple serine-like proteases from *Amycolatopsis orientalis* spp. *orientalis* degrade high-molecular-weight PLA film and casein (Li *et al*., 2008). However, our results indicated that the purified enzyme from *L. sacchari* LP175 degraded ester bonds of high-MW PLLA rather than those of short peptides and Ala-Ala.

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Table 25 Substrate specificity on the purified PLLA-degrading activity

Substrate	Specific activity (U/mg of protein)
Polyester	
PLLA	388
PCL	20
Protein	
Casein	340
Gelatin	262
Suc-$(Ala)_3$-*p*NA	38
p-Nitrophenol ester	
*p*NP-acetate (C2)	0.0
*p*NP-butyrate (C4)	0.0
*p*NP-decanoate (C10)	0.0
*p*NP-palmitate (C16)	0.0
*p*NP-stearate (C18)	0.0
Olive oil	0.0

5.3 Effects of pH and temperature on the stability and activity of PLLA-degrading activity

The optimal pH and temperatureof purified PLLA-degrading enzyme were determinedover a range from 3.5 to 12 and 30 to 100 ^{o}C, respectively. The highest activity of PLLA-degrading enzyme was recorded at 60 ^{o}C and pH 9.0. In regard to investigation of the pH and temperature stability (Figure 26), enzyme activity was stable at pH 8.5–10.5 and maintained 88% of relative activity at pH 12.0. The enzyme maintained high activity in a temperature range of 35–50 ^{o}C for 24h. However, the relative activity remained at 80.3 % at 60 ^{o}C. These results were similar to previous findings of optimum pH (9.0 to 10.9) and temperature (37 to 70 ^{o}C) for PLLA-degrading enzymesproduced by most microorganisms (Li *et al*., 2008; Nakamura *et al*., 2001; Sakai *et al*., 2001; Sukkhum *et al*., 2009b).

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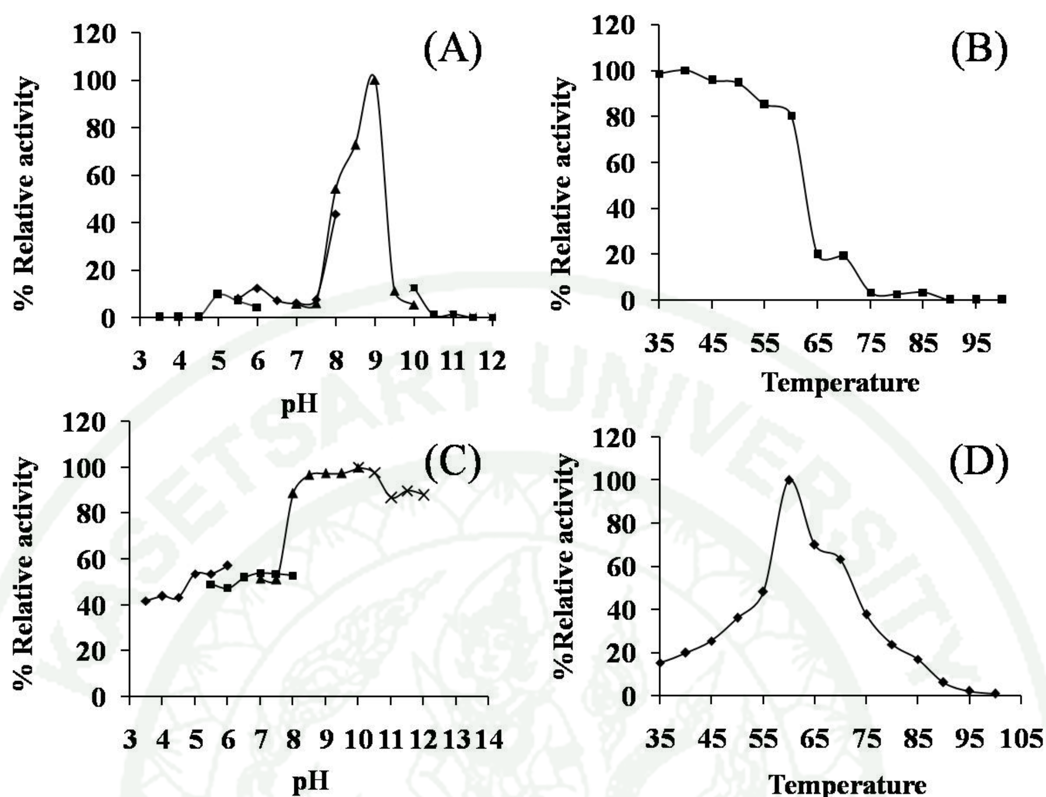

Figure 26 Effects of pH and temperature on purified PLLA-degrading activity. Optimum pH and pH stability (A and C). Optimum temperature and thermostability (B and D)

5.4 Effect of detergents, inhibitors and metal ion of purified PLLA-degrading enzyme

The effect of different class-specific protease inhibitors, such as metalloproteinase, serine/cysteine and thiol proteinase (thiolendopeptidase orthiolprotease), on purified PLLA-degrading activity is shownin Table 26. Enzyme activity was completely inhibited by PMSF, aclass-specific serine protease inhibitor. Enzyme activity was strongly inhibited by 1–10mM EDTA and EGTA. The activity was also decreased by 5.23 %, 86.77 % and 90.09 %, in the presence of 1, 5 and 10 mM 1,10-phenanthroline, respectively. The class-specific serine/cysteine and thiol protease inhibitors such as DTT and β-ME failed to inhibit enzyme activity.

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Table 26 Effect of various inhibitory substances of PLLA-degrading activity on purified enzyme.

Inhibitor	Concentration (mM)	% Relative activity
Control		100
EDTA	1	6.2 ± 0.11
	5	5.5 ± 0.22
	10	5.3 ± 0.01
EGTA	1	0.7 ± 0.24
	5	0.3 ± 0.08
	10	0.5 ± 0.00
1,10-Phen	1	94.8 ± 1.19
	5	13.2 ± 1.07
	10	9.9 ± 2.60
1,10-Phen	1	94.8 ± 1.19
	5	13.2 ± 1.07
	10	9.9 ± 2.60
PMSF	1	5.5 ± 0.05
	5	4.6 ± 0.71
	10	4.5 ± 0.28
DTT	1	78.7 ± 0.16
	5	87.9 ± 2.65
	10	86.5 ± 0.28

EDTA, Ethylenediaminetetraacetate; EGTA, ethylene glycol tetraacetic acid; 1,10-Phen, 1,10-phenanthroline; PMSF, phenylmethylsulfonylfluoride; DTT, dithiothreitol

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The residual activity of purified PLLA-degrading enzyme was assayed in the presence of detergents such as CTAB, SDS, Tween 20, Tween 40, TritonX-10 and PlysurfA210G, and reducing agents such as urea (Table 27). The activity of PLLA-degrading enzyme was strongly inhibited when incubated with SDS, Tween20, Tween 80, and Plysurf A210G. A loss of enzyme activity was exhibited in the presence of CTAB and Triton X-10:64.6 % and 69 % activity at a final concentration of 0.1 % (w/v), respectively. Raising the concentration of urea from 1 to 5% had noeffect on enzyme activity, which remained constant at approximately 86 % (1–10 mM).

The effect of metal ions under standard assay is shown in Table 28. The purified PLLA-degrading activity was treated with EDTA to a final concentration of 5 mM; EDTA was then removed by dialysis.The relative activity of the enzyme was more than 100% of the control level (119.56 %) after adding1mM Co^{2+} ions; but adding 4 mM Co^{2+} ions resulted in inactivation. The relative enzyme activity was greater than 80% of the control level in the presence of 1mM $Ca^{2+}$and Cu^{2+} ions, but was strongly inhibited at high concentration (4 mM). Enzyme activity was also strongly inhibited when incubated with Fe^{2+}, Mg^{2+}, Zn^{2+}. In particular, adding EDTA (at a final concentration of 5 mM) back into the reaction mixture in the presence of 1mM and 4mM of all cations did not affect enzyme activity. These results were similar to those of thermostable alkaline protease from *Bacillus* sp.(Patil *et al*., 2010) and PLLA-degrading serine protease from *Actinomadura* sp.T16-1 (Sukkhum *et al*., 2009b).

The specific inhibition profile of the purified PLLA-degrading enzyme succeeded to inhibit serine/cysteine proteinase (PMSF) and all serine protease (Plysurf A210G), but failed to inhibit thiolproteinase (DTT and β-ME) and metalloproteinase (1 mM 1,10-phenanthroline), indicating that it was a serine proteinase. Although EDTA and EGTA are strong inhibitors of metalloproteinase, much of the inhibition of hyperthermophilic serine protease was considered to be due to the loss of activity from enzyme denaturation rather than inhibition of enzyme molecules by removing Ca^{2+} ions from the enzyme, leading to the loss both of stabilizing Ca^{2+} ions and activity when treated with EDTA and EGTA (Catara *et al*., 2003; Peek *et al*., 1992). Furthermore, EDTA is not a good indicator for a

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metalloproteinase inhibitor because a large number of other enzymes require calcium for activity and EDTA is an excellent calcium chelator (Powers *et al*., 1986). Normally, 1,10-phenanthroline isa powerful zinc-metalloproteinase inhibitor and is used as thestrongest indicator for a classical metalloproteinase inhibitor. Although, enzyme activity was not inhibited by 1,10-phenanthroline at low concentrations of 1 mM,it was inhibited at high concentrations of 5 and 10mM, suggesting that 5 mM or more 1,10-phenanthroline enhances the inhibitory activity. Plysurf A210G has been reported to inhibit only serine protease at high concentrations but not other proteases (Lim *et al*., 2005). In addition, this enzyme has a strong resistance to urea and β-ME, which is related to thermostable serine protease in *Thermoanaerobacter tengcongensis* (Koma *et al*., 2007). The resistance toward urea is a common property of thermostable serine/alkaline protease, such as from the*Thermoactinomyces* strain from hot springs (Aksoy *et al*., 2012). Furthermore, the resistance of the enzyme to urea and β-ME might indicate that hydrogen bonds play little part in enzyme stabilization, and that disulfide-like bonds are not involved in preservingthe enzymatic structure (Croocker *et al*., 1999; Sako *et al*., 1997).

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Table 27 Effect of reagents on purified PLLA-degrading enzyme

Reagent	Concentration	% Relative activity
CTAB	0.1%	35.8 ± 0.53
	0.2%	34.0 ± 0.29
	0.5%	15.4 ± 1.84
SDS	0.1%	1.2 ± 0.17
	0.2%	1.7 ± 0.09
	0.5%	1.7 ± 0.29
Tween20	0.1%	0.1 ± 0.00
	0.2%	0.1 ± 0.01
	0.5%	0.1 ± 0.00
Tween80	0.1%	1.2 ± 0.24
	0.2%	1.3 ± 0.15
	0.5%	1.4 ± 0.00
Tritron X-10	0.1%	31.0 ± 0.00
	0.2%	30.8 ± 0.10
	0.5%	29.8 ± 0.00
Plysurf A210G	0.1%	1.4 ± 0.00
	0.2%	3.5 ± 0.53
	0.5%	6.9 ± 0.53
Urea	1 mM	86.9 ± 3.41
	5 mM	85.0 ± 0.76
	10 mM	86.4 ± 3.80
β-mercaptoethanol	1 mM	86.6 ± 1.14
	5 mM	86.1 ± 0.76
	10 mM	84.5 ± 1.14

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Table 28 Effect of metal ions on purified enzyme activity

Metal ion	Concentration (mM)	% Relative activity	
		Treated EDTA	Treated EDTA
Control		100 ± 0.09	45.8 ± 0.15
Ca^{2+}	1	82.5 ± 0.04	0.3 ± 0.06
	4	15.3 ± 0.09	0.5 ± 0.04
Co^{2+}	1	119.6 ± 0.23	13.0 ± 0.04
	4	55.2 ± 0.51	1.1 ±0.00
Fe^{2+}	1	45.6 ± 1.51	2.6 ± 0.1
	4	20.0 ± 0.54	0.0 ± 0.0
Mg^{2+}	1	45.5 ± 0.49	1.4 ± 0.15
	4	10.9 ± 0.08	1.2 ± 0.08
Mn^{2+}	1	67.7 ± 0.15	5.2 ± 0.15
	4	0.0 ± 0.00	5.8 ± 0.02
Zn^{2+}	1	35.1± 0.51	2.0 ± 0.17
	4	19.7 ± 0.21	4.8 ± 0.02
Cu^{2+}	1	91.6 ± 0.15	1.0 ± 0.27
	4	0.7 ± 0.00	1.6 ± 0.04
Na^{+}	1	64.9 ± 0.02	5.7 ± 0.00
	4	46.2 ± 0.19	1.3± 0.07
K^{+}	1	64.2 ± 0.25	9.5 ± 0.03
	4	47.0 ± 0.02	0.0 ± 0.00

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6.5 N-terminal amino acids sequence analysis of purified PLLA-degrading enzyme

Analysis by SDS-PAGE (Figure 27) and gel filtration chromatography on a Sephacryl S-100 column indicated that the molecular weight of PLLA-degrading enzyme from strain LP175 was 27.8 and 28.7 kDa, respectively, which was very close to that of thermitase from *Thermoactinomyces vulgaris* (MW=28.369)(Meloun*et al*., 1985). The N-terminal amino acid sequence from *L. sacchari* LP175 was analyzed by an automatic protein sequencer. The first 15 amino acid residues of the N-terminus of *L. sacchari* LP175 were the sequence Tyr-Thr-Pro-Asn-Asp-Pro-Tyr-Phe-Ser-Ser-Arg-Gln-Tyr-Gly-Pro. The result of BLAST analysis elucidated 100% homology with thermostable serine protease (thermitase) from *Thermoactinomycesvulgaris.* A comparison of the N-terminal amino acid sequences of enzyme serine protease, capable of degrading PLLA, from strains belonging to bacteria in the order *Actinomycetales*found Pro at position 6 in amino acid residues(Nakamura *et al*., 2001; Pranamuda *et al*., 2001; Sakai *et al.,*2001;Sukkhum *et al*., 2009).

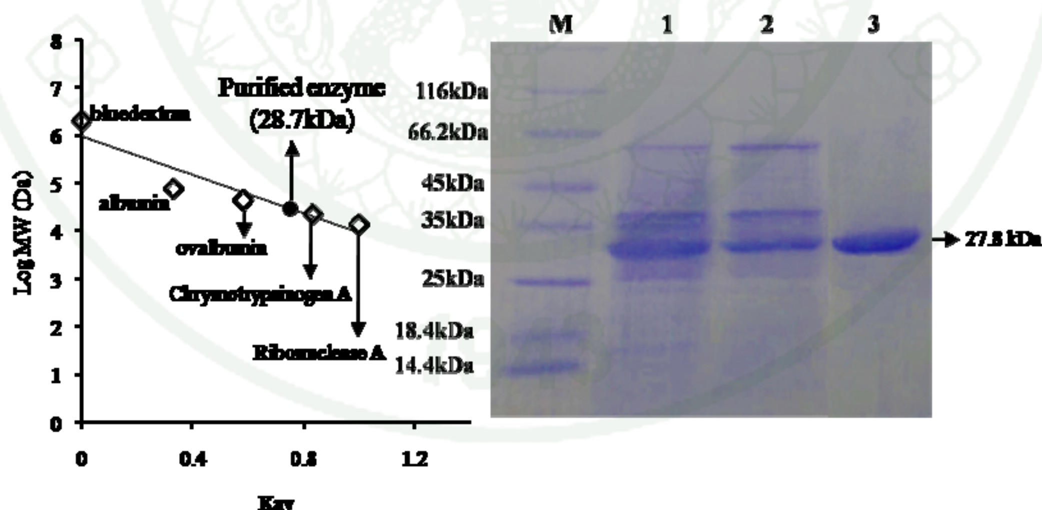

Figure 27 Determining molecular weight of PLA-degrading enzyme purified from *L. sachari* LP175 was determined by using gelfiltration chromatography (left) and SDS-PAGE (right). (M , Marker; 1, culture supernatant; 2, dialyzed culture supernatant; 3, CM-Sepharose)

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5.6 Comparision of characterization of serine protease from *L. sacchari* LP175 and *Thermoactinomyces vulgaris*

The properties of serine protease from *L. sacchari* LP175 – such as optimum pH and temperature, thermal and pH stability, substrate specificity, inhibition sensitivity, molecular weight, and N-terminal amino acid sequence – were similar to those of thermostable serine protease (thermitase) from *Thermoactinomyces vulgaris*. The data shown in Table 29 indicate that this enzyme is a serine protease (thermitase). Howevr, the properties of *L. sacchari* LP175 differs in metal ion perperties. *Thermoactinomyces vulgaris* was strongly inhibiyed with only $Hg^{2\ but}$ *L. sacchari* LP175 inhibited with three metal ions (Fe^{2+}, Mg^{2+}, Zn^{2+}).

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Table 29 Comparision of characterization of thermostable serine protease from *L. sacchari* LP175 and *Thermoactinomyces vulgaris*

Characteristic	*L. sacchari* LP175	*Thermoactinomyces vulgaris*
Molecular weight (kDa)	27.8 (SDS-PAGE) 28.7 (Gelfitration)	28.369 kDa [a]
Optimum Ph	9.0	7.5 and 9.5 [c,b]
pH stability	8.5-10.5	6.0-7.5 [c]
Optimum temperature (°C)	60	60-75[d]
Temperature stability (°C)	35-50	-
Substrate specificity	PLA, casein, gelatin	Elastin, collagen [c] Gelatin, casein, field bean protein, serum albumin, gluten[d]
Inhibitor	PMSF, EDTA, EGTA	PMSF [b]
Metal ion		
Stimulation	Co^{2+}	-
Weak inhibition	Ca^{2+}, Cu^{2+}	-
Modera inhibition	K^{+},Na^{+}, Mn^{2+}	-
Strong inhibition	Fe^{2+}, Mg^{2+}, Zn^{2+}	Hg^{2+} [b]
N-terminal amino acid residues	Tyr-Thr-Pro-Asn-Asp-Pro-Tyr-Phe-Ser-Ser-Arg-Gln-Tyr-Gly-Pro	Try-Thr-Pro-Asn-Asp-Pro-Tyr-Phe-Ser-Ser-Arg-Gln-Tyr-Gly-Pro [a]

-, not determinded

[a] , Meloun *et al.* (1985); [b], Kleine and Kattmann (1982); [c], Kleine (1982); [d] Bhnke *et al.* (1978)

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CONCLUSION AND RECOMMENDATION

Conclusion

A total of 11 strains were isolated from forest soils, and showed the ability to degrade PLLA on an agar plate and in culture broth.Phylogenetic study by 16S rRNA sequence analysis of PLLA-degrading thermophilic bacteria indicated that strainsLP157 was identified as *L. sacchari*in the family *Thermoactinomycetaceae*. ·Among the isolates, *L. sacchari* LP157 showed the highest PLLA-degrading activity. The fermentation process for PLLA-degrading enzyme production by thermophilic PLLA-degrading *L. sacchari* LP175 was investigated. Comparison of the PLLA-degrading enzyme production in SSF and SmF using two agricultural wastes found that SmF (5.18 U/ml) gave a higher enzyme activity than SSF (2.74 U/ml). The optimal concentration of cassava chip, soybean meal and PLLA powder obtained by statistical analysis using response surface methodology (RSM) for the maximum production of PLLA-degrading enzyme was 0.464% cassava chip, 0.153% soybean meal and 0.031% PLLA powder, respectively. Under these conditions, the model predicted PLLA-degrading activity of 68.5 U/ml. Verification of the optimization showed that PLLA-degrading enzyme production of 65.5 U/ml was observed in a shake flasks experiment. Observing the PLLA-degrading production in the 3-L airlift fermenter found that the PLLA-degrading activity increased up to 94.4 U/ml within 18 h cultivation. The effect of physical parameters, pH, aeration rate and temperature on PLLA-degrading enzyme production by *L. sacchari* LP175 in a 3L airlift fermenter with 2L working volume was performed. The conditions for optimal aeration rate, temperature and pH for PLLA-degrading enzyme production was 0.5 vvm aeration rate, temperature at 50°C and pH 7.0. To our knowledge, this is the first report of low medium cost for PLLA-degrading enzyme production by *L. sacchari* LP175.

The purified PLLA-degrading enzyme from *L. sacchari* LP175 by CM Sepharose chromatography showed the ability to degrade casein and gelatin. Optimum pH and temperature were 7.0 and 60°C, respectively. The enzyme activity exhibited resistance to urea and β-ME, suggesting that the hydrogen bonds play little

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part inenzyme stabilization, and that the disulfide bondsare not involved in preserving the enzymatic structure.The properties of the purified enzyme and N-terminal sequencingwere similar to those of thermostable serine protease from *Thermoactinomyces vulgaris*. It is suggested that this purified enzyme was serine protease that can degradethe ester bonds of high-MWPLLA.

Recommendation

1. The development of PLLA-degrading enzyme produced by this strains in different method should be studied.

2. The application of characterizatic PLLA-degrading enzyme produced by strain LP175 should be studied for testing of bioplastic type.

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APPENDIES

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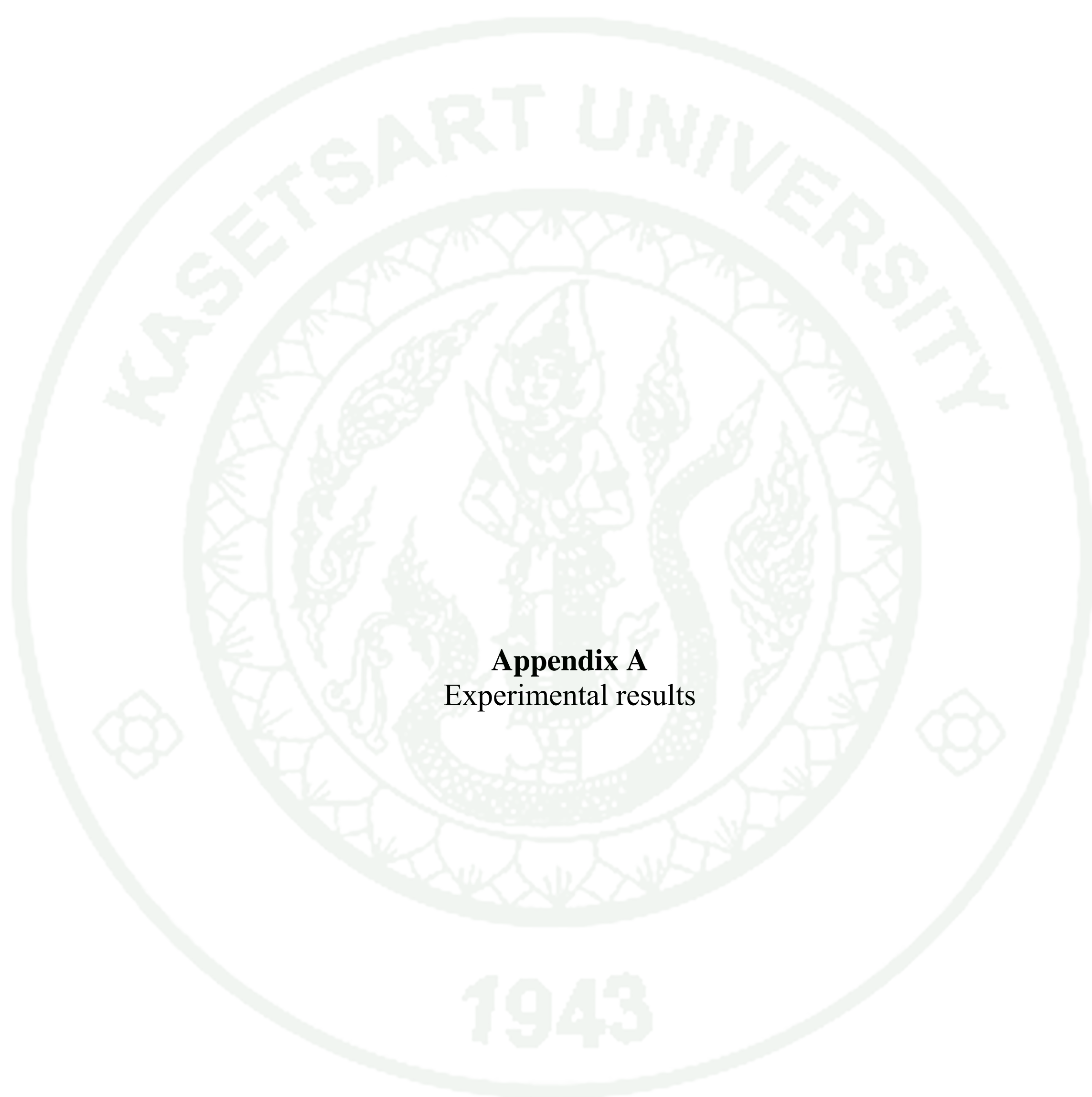

Appendix A
Experimental results

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Protein sequence report

Created on 13-Apr-12

Alta Bioscience code: S5551

Customer sample code: LP 175

N terminus

Residue			
1	Y		
2	T		
3	P		
4	N		
5	D		
6	P		
7	Y		
8	F		
9	S		
10	S		
11	R		
12	Q		
13	Y		
14	G		
15	P		

Comments:-

Notes on the presentation of the data:-
? = most probable assignment. - = nothing detected at this position. X = unknown component
Where several sequences are observed, an attempt is made to arrange them in descending order of abundance at each residue. However because of difficulties inherent in the sequencing process, this should be treated as a guide only.

Reported by ____________________ Date ___/___/___

File name: S5551 S hanphakphoom.doc
Template: Proseq15, version 4. Last modified 14th March 2012

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Appendix Figure A1 15 amino acids sequencing of serine protease from *L. sacchari* LP175

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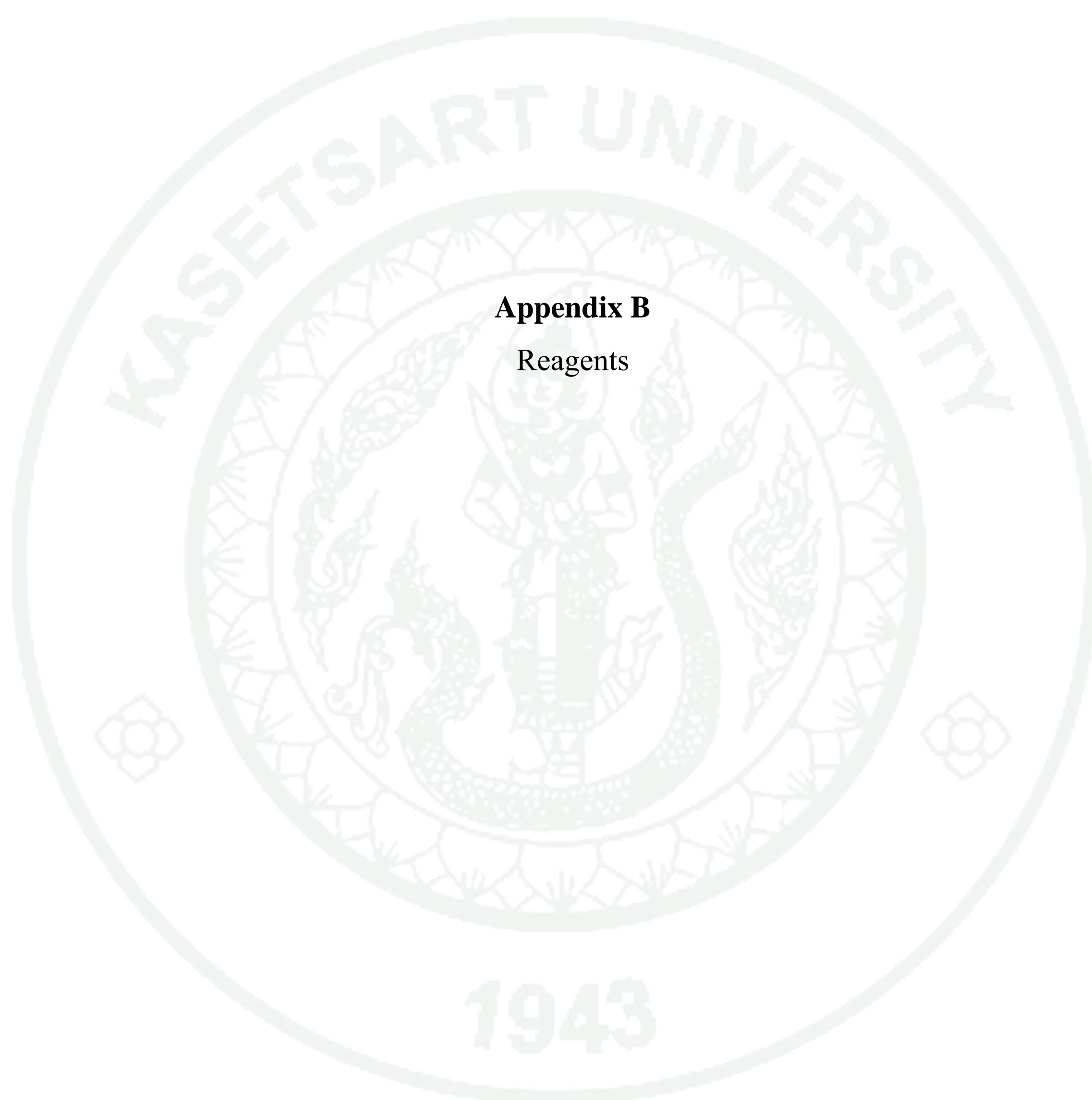

Appendix B

Reagents

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Appendix B1 media composition and preparation

Basal medium (Sukkhum *et al*., 2009a)

ISP medium no.2 Yeast extract-malt extract agar

Yeast extract	4	g
Malt extract	10	g
Dextrose	4	g
Distilled water	1	L
Agar	20	g
Adjust to pH 7.3		

Liquefy agar by steaming at 100ºC 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 B2 Composition of reagents of DNA extraction was as follows:

1. TE buffer :
   10.8 g Tris-HCl
   0.83 g EDTA-2Na
   pH was adjusted to 8.0 with HCl
   Add distilled water to 1,000 ml

2. TAE buffer (10X)
   12.1 g Tris-base
   2.9 ml 100% acetic acid
   5 ml 0.5 M EDTA (pH 8.0)
   Add distilled water to 250 ml

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3. SET buffer:	75 mM NaCl,
	25 mM EDTA pH8
	20 mM Tris-HCl pH 7.5
4. Lysozyme	50 mg/ml in water
5. Proteinase K:	20 mg/ml in water
6. 10% SDS	
7. 5M NaCl	
8. Chloroform	
9. isopropanol	
10. 70% ethanol	

Appendix B3 Composition of reagents of agarose gel electrophoresis was as follows:

1. Loading dye	1.25 g Bromophenol blue
	1.25 g Xylene cyanol FF
	15 g Glycerol
	Add distilled water to 50 ml
2. 1% agarose gel	0.8 g Agarose
	80 ml TBE buffer/TAE buffer
	10 mg/ml Ethidium bromide 4 µl
3. TBE buffer (10x)	27 g Tris-base
	13.75 g H_3BO_3 (Boric acid)
	2.32 g EDTA-2Na

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Add distilled water to 250 ml

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

The enzyme was precipitated by adding equal vol. of colded 10% (w/v) Trichloroacetic acid (TCA). The solution was centrifuged at 10,000 rpm for 5 min. 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 0.96M 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 (adjust before use)

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

1. Stain:	450 ml water 500 ml methanol 75 ml acetic acid 5 g Coomassie brilliant blue
2. Destain I:	1.0 L water 1.0 L methanol 200 L acetic acid
3. Destain II:	150 L methanol 225 L acetic acid bring to 1.0 L with water

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Hanphankphoom, S., N. Maneewong, S. Sukkhum, S. Tokuyama and V. Kitpreechavanich. 2010. Purification and characteristics of PLA-degrading enzyme produced from *Laceyella sacchari* LP175. 19-21 Nov. 2010. p. 56. **The 2nd Joint Seminar in Asian Core Program Khon Kaen, Thailand.**

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