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Full Length Research Paper

A novel poly (L-lactide) degrading thermophilic actinomycetes, *Actinomadura keratinilytica* strain T16-1 and *pla* sequencing

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An actinomycete strain T16-1 demonstrated the maximum poly (L-Lactide)-degrading activity when cultured in basal liquid medium at 50°C. According to 16S rDNA sequence analysis, chemotaxonomic and DNA-DNA hybridization revealed that strain T16-1 belong to the family Thermomonosporaceae, genus *Actinomadura*. On the basis of phenotypic and phylogenic data, strain T16-1 which is a novel PLA-degrading thermophilic actinomycete was identified as *Actinomadura keratinilytica*, but the color of its colony on ISP plates, NaCl tolerant and utilization of mannitol, raffinose and arabinose were different. Partial sequence of poly (L-lactide) depolymerase gene from strain T16-1 was demonstrated. The gene consisting of 222 amino acids was related to serine protease from *Streptomyces* sp. with 43 to 46% identity.

Key words: *Actinomadura keratinilytica,* cloning, identification, poly (L-lactide).

INTRODUCTION

Abundant quantities of plastic wastes are serious problems for global environment and have stimulated the improvement of biodegradable plastics. Poly (L-lactide) (PLA) aliphatic polyester is synthesized from L-lactic acid, which can be produced from farm and agricultural products such as cassava, rice, corn and corncob by fermentation methods (Miura et al., 2004; Wee et al., 2006). Recently, there has been interest in using plastic composting by microbes as a method for treating plastic waste (Tomita et al., 2003).

Several thermophilic bacteria such as *Bacillus brevis*, *Bacillus stearothermophilus and Geobacillus thermocatenulatus* have been reported to possess PLA-degrading activity (Tomita et al., 1999, 2003, 2004). Some actinomycetes, belonging to members of the family Pseudonocardiaceae were also able to produce enzymes that decompose PLA, such as *Amycolatopsis* sp., *Lenzea waywayandensis* and *Kibdelosporangium aridum* (Jarerat et al., 2002). Recently, PLA-degrading enzyme production by *Actinomadura* sp. using statistical method was reported by Sukkhum et al. (2009a). Unculturable microorganisms were identified from the compost consisting of PLA by using metagenomic method including *Paecilomyces*, *Thermomonospora* and *Thermopolyspora* (Sangwan and Wu, 2008).

In order to screen novel microorganism that are suspected to possess PLA-degrading activity at high

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temperature for composting application and recycling of biodegradable plastic wastes, PLA-degrading actinomycetes were isolated at 50°C. The strain that produced the highest PLA degrading activity was selected and identified.

This is the first finding of a strain belonging to the species *Actinomadura keratinilytica* with the capacity to degrade PLA, producing appropriate enzyme at 50°C. The *pla* sequencing of a new PLA-degrading strain was investigated in this study.

MATERIALS AND METHODS

Isolation of PLA-degrading actinomycetes and their enzyme production

Soil samples (0.1 g each) were suspended in 9 ml sterile distilled water mixed well and then plated on an emulsified PLA agar plates. The plates were prepared as follows. 1 g PLA pellet was dissolved in 40 ml dichloromethane. To 1,000 ml of a basal medium (containing per liter, $(NH_4)_2SO_4$, 4 g; K_2HPO_4, 2 g; KH_2PO_4, 1 g; $MgSO_4.7H_2O$, 0.5 g; yeast extract, 1 g; agar, 15 g, pH 7.0; sterilized at 121°C for 15 min), 40 ml of the PLA solution was added. The medium was sonicated with an ultrasonic processor model VCX 500 (Sonic and Materials, INC., Newtown, USA) for 5 min. The colonies forming clear zones were selected as PLA-degrading strains and were sub-cultured and purified on an emulsified PLA agar plate.

The ability of isolates on the enzyme production was determined in culture broth. The strains were cultured in 250 ml Erlenmeyer flask containing 100 ml of basal liquid medium consisting per liter, PLA film, 1 g; $(NH_4)_2SO_4$, 4 g; K_2HPO_4, 2 g; KH_2PO_4, 1 g; $MgSO_4.7H_2O$, 0.5 g; extract, 1 g, pH 7.0; with shaking speed of 150 rpm for 4 days at 50°C. The culture broth was centrifuged and the obtained supernatant was used to analyze the PLA-degrading activity. PLA-degrading enzyme activity was assayed based on decrease in turbidity by a modified method of Nakamura et al. (2001) as described by Sukkhum et al. (2009a). One unit of the PLA-degrading activity was defined as a 1 unit decrease in optical density at 630 nm per min under the assay condition described.

16S rDNA sequencing and G+C content analysis

For biomass preparation, strain T16-1 was grown in shake flask of tryptone soya broth (TSB) at 50°C for 2 to 3 days and harvested by centrifugation. Cells for the chemotaxonomic analysis were washed twice in distilled water and freeze-dried. Cells for the molecular systematic analyses were washed in NaCl-EDTA buffer (0.1M EDTA, pH 8.0, 0.1M NaCl) and stored at -20°C until required.

Genomic DNA was extracted from strain T16-1 as described by Hopwood et al. (1985). Further purification steps including RNase treatment were carried out according to the method of Saito and Miura (1963). The G+C content of the DNA was determined by the HPLC method of Tamaoka and Komagata (1984). A gene fragment specific for 16S rRNA gene-coding region was amplified by means of PCR. Two primers, 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTGATCCAGCC-3') were used. All primer positions were specified by the *Escherichia coli* numbering system (Brosius et al., 1978). The amplified and purified 16S rRNA genes were sequenced directly with a Big Dye terminator V3.1 Cycle sequencing Kit on an ABI 3100 automated DNA sequence (Applied Biosystems, Foster City, Califonia, USA).

Multiple alignments of the sequences obtained were performed using program CLUSTAL X (version 1.81) (Thompson et al., 1997) Gaps and ambiguous bases were eliminated. Comparison of the aligned sequences was made for 1,254 bases of 16S rRNA gene sequence in constructing phylogenetic trees by the neighbor-joining method of Saitou and Nei (1987) using the MEGA 3 program (Kumar et al., 2004). Distance matrices for the aligned sequences were calculated by the two-parameter method of Kimura (1980). Robustness for individual branches was estimated by bootstrapping of 1,000 replications (Felsenstein, 1985).

DNA-DNA hybridization

DNA-DNA hybridization experiments were used to resolve the taxonomic relationships between representatives of these closely related species. Levels of DNA relatedness of strain T16-1 and related organisms was determined with a method modified from that of Ezaki et al. (1989), using the β-galactosidase/4-methylumbelliferyl-β-galactoside system.

Chemotaxonomic characterization

The isomer of diaminopimelic acid in the cell wall and the whole-cell sugar pattern were determined as described by Staneck and Roberts (1994). Menaquinones were analysed by LC/MS (Shimadzu LC-MS application data sheet No. 010). Phospholipids were extracted by the method of Minnikin et al. (1979) and identified using two dimensional TLC. Cellular fatty acid methyl esters were prepared and analysed according to the protocol of the MIDI Sherlock Microbial Identification System (Agilent Technologies 6890N Network GC System) (MIDI, 2002; Sasser, 1990).

Cultural and morphological characterization

To examine the extent of growth, pigmentation and color of colony, strain T16-1 was grown on inorganic salt-starch agar (International Streptomyces Project (ISP-4) as described by Shirling and Gottlieb (1966). Morphological structures were studied using 20 days of strain on humic vitamin agar (HV) and observed by a scanning electron microscope (model JSM6060, JEOL, Ltd., Tokyo, Japan).

Phenotypic characterization

In addition to PLA degradation, strain T16-1 was also examined for the ability to degrade cellulose (cellulose medium), gelatin (glucose-peptone-gelatin medium), skim milk (skim milk medium) and starch (ISP-4) as described by Hamada (2000), in addition to PLA. NaCl tolerance and growth temperature were assessed using yeast extract-malt extract medium (ISP-2). Utilization of carbon sources was tested by using carbon utilization medium (ISP-9) modified from Pridham and Gottlieb (1948). Each carbon source was added to ISP-9 medium to give a final concentration of 1% (w/v). Lactic acid utilization was also tested. The basal medium (Tomita et al., 2003) with 0.1 % lactic acid was used in this test. The results were taken by measuring dry cell weight.

Sequencing of *pla* from strain T16-1

Bacterial strains and plasmid

Strain T16-1 was used as chromosomal DNA sources. Genomic DNA was extracted from strain T16-1 as described by Hopwood et al. (1985). *E. coli* JM109 was used as the host for recombinant

plasmid. Plasmid pUC19 was used as vector containing *pla*.

Preparation of oligonucleotide primers for cloning of *pla* from strain T16-1

N-terminal amino acid sequence (NPPSAGLDR) of purified PLA-degrading enzyme was used for design forward primer, plaF (5'-CCSWCSGCSGGSCTSGACCG3-'). According to Blast p search, reverse primer was obtained from the most closely related amino acid sequence, plaR (5'-GGSGTSGCCATSGWSGTSCC-3'). In the sequences, S represent C or G and W represent A or T. After PCR amplification, a ca. 650 bp fragment was obtained from genomic DNA of strain T16-1 using Takara Ex Taq polymerase.

Cloning of *pla* and Its DNA sequencing

Vector (pUC19) was digested by *Sma*I and ligated with *pla*. The plasmid containing *pla* was designed as pUCpla. Plasmid pUCpla was introduced into protoplast of *E. coli* JM109, a recombinant *E. coli* strain containing *pla* was selected by blue/white colony on Luria-Bertani (LB) medium containing 10 mg/ml ampicillin, IPTG and X-gal. Recombinant plasmid (pUCpla) was extracted according to nucleospin plasmid kit and direct sequence of multiple containing site (MCS) by using two oligonucleotide primers, M13F (5'-GTAAAACGACGGCCAG-3') and M13R (5'-GGAAACAGCTATGACCATG-3').

Protein and nucleotide sequences were compared with those on databases using FASTA (version 3.0) and BLAST (version 1.49) programs implemented at the EMBL/GenBank/DDBJ nucleotide sequence databases. Multiple-sequence alignment was done using a GENETYX program (Software Development Co., Tokyo, Japan).

RESULTS

Isolation of PLA-degrading actinomycetes and their PLA degrading activity

Eighty samples of surface layer soil were taken from Huai kha khaeng wildlife sanctuaries, Uthaithani province, Thailand. However, among these soil samples, only 10 samples were found 11 active strains isolated at 50°C and 2 isolates at 40°C. This indicates that PLA-degrading microorganisms could be regarded as having a lower population in the soil as reported by Pranamuda et al. (1997) and Ikura and Kudo (1999).

Among these, strain T16-1 produced the highest PLA-degrading activity at 22 U/ml, while, strains T16-4 and T9-1 produced 15 and 10 U/ml, respectively. The clear zone formation on emulsified PLA agar and enzyme activity in culture broth did not show a strong correlation, but some strains which had high activity might have a larger clear zone. Beside the size of clear zone, strains T16-1, T16-4, T9-1 and T7-1 demonstrated high clearness on the plate, which could associated with the extent of their activity in culture broth.

The GenBank accession number for the 16S rRNA sequence of strain T16-1 is FJ199994. The strain T16-1 was deposited in culture collection as BCC 28970 and NBRC 104111.

Genotypic properties

For classification, strain T16-1 was subjected to a polyphasic investigation. In a phylogenetic tree based on 16S rRNA gene sequences for 1,254 nucleotides (nt), strain T16-1 clustered with the members of the genus *Actinomadura* (Figure 1). The highest levels of 16S rRNA gene sequence similarity was found to be *Acinomadura keratinilytica* WCC-2265^T (100%), *Actinomadura rubrobrunea* NBRC 15275^T (98.0%) and *Actinomadura viridilutea* NBRC 14480^T (97.9%).

In the present investigation, it was evident that DNA–DNA relatedness values in reciprocal hybridizations were much lower than 70%, indicating that bacterial strain represents a separate genomic species. In the case of strain T16-1 and related species: [*A. keratinilytica* WCC-2265^T (80.1 to 86.5%), *A. viridilutea* NBRC 14480^T (30.3 to 33.2%) and *A. rubrobrunea* NBRC 15275^T (34.4-36.6%)], strain T16-1 should be identified as *A. keratinilytica*. The G+C content of the DNA was 72.2 mol%.

Chemotaxonomic characterization of Strain T16-1

Strain T16-1 contained *meso*-diaminopimelic acid. Galactose, glucose, madurose, mannose and ribose were detected in whole-cell hydrolysates indicating a chemotype IIIB cell wall (Lechevalier and Lechevalier, 1970). Polar lipids of strain T16-1 included diphosphatidylglycerol, phosphatidylinositol, phosphatidylglycerol and phosphatidylinositol mannosides. 14-Methyl-pentadecanoic acid (16:0 iso; 23% of total fatty acids) and 15-methyl-hexadecanoic acid (17:0 iso; 30% of total fatty acids) were the major fatty acids (Table 1).

WCC-2265^T also produced a relatively large proportion of iso-branched fatty acids, predominantly 16:0 iso and 17:0 iso. The major menaquinones of strain T16-1 were MK-9(H_6) (61%), MK-9(H_4) (18%) and MK-9(H_8) (17%). In addition, a small amount of MK-9(H_2) was detected. Fatty acid, DAP type and menaquinones of strain T16-1 was the same as strain WCC-2265^T (Puhl et al., 2009). The results summarized in Table 1 suggest that strain T16-1 belonged to the genus *Actinomadura* sp.

Cultural and morphological characteristics

The phenotypic properties of strain T16-1 and WCC-2265^T was shown in Table 2. Strain T16-1 exhibited good growth on ISP-2, ISP-3 and ISP-4. The substrate mycelium of the strain T16-1 was cream-yellow. The aerial mycelia were rare but when present green on ISP-4. This was different from substrate mycelium of WCC-2265^T which was yellow-orange on ISP-2 and gray-white on ISP-3 and ISP-4. Morphological observation of a 20-d-

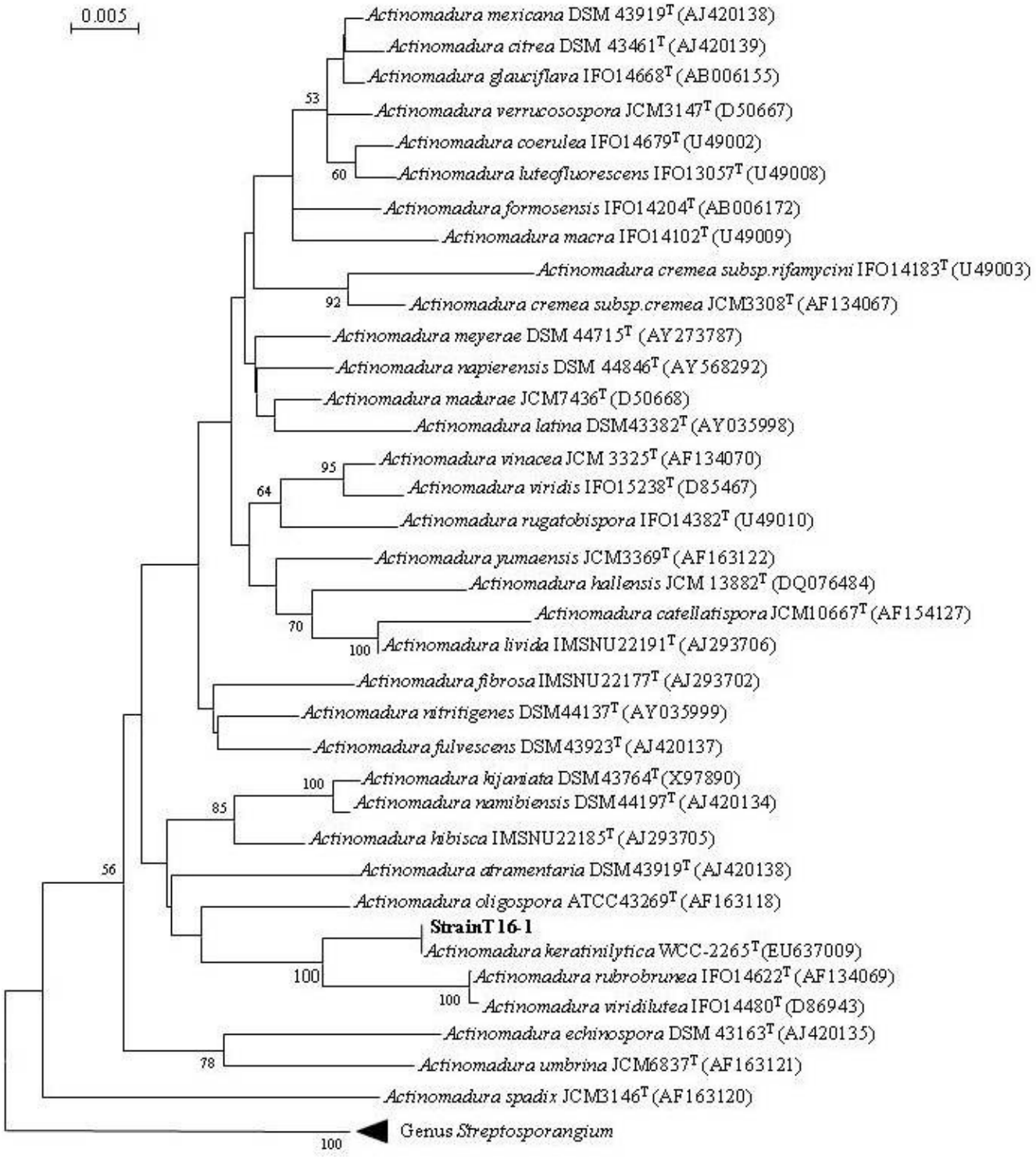

Figure 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing relationships of strainT16-1 to representatives of validly described species of *Actinomadura*. Bootstrap values at branching points are expressed as percentages from 1000 replications (only values greater than 50% are indicated).The scale bar indicates 0.005 substitution per nucleotide position. T, type strain.

old culture of the strain grown on Humic vitamin (HV) agar revealed the presence of oligosporic curved chains of spiny spores.

Phenotypic properties of strain T16-1

Strain T16-1 utilized D-glucose, inositol, raffinose,

Table 1. Chemotaxonomic characteristic of strain T16-1.

Characteristic	Strain T16-1
DAP type	*meso*-DAP
Sugar type	Type IIIB (Galactose, glucose, madurose, mannose and ribose)
Phospholipid	Diphosphatidylglycerol, phosphatidylinositol, phosphatidylglycerol, phosphatidylinositol mannosides
Menaquinone	
MK-9(H_6)	61%
MK-9(H_4)	18%
MK-9(H_8)	17%
MK-9(H_2)	small amount
Fatty acid*	
15:0 iso	14.8%
15:0	6.7%
16:0 iso	22.8%
16:0	7.3%
17:0 iso	29.6%
17:0	13.6%
17:0 10 methyl	5.3%

*Abbreviations for fatty acids: 15:0 iso, 13-methyltetradecanoic acid; 15:0, pentadecanoic acid; 16:0 iso, 14-methylpentadecanoic acid; 16:0, hexadecanoic acid; 17:0 iso, 15-methyl hexadecanoic acid; 17:0, heptadecanoic acid; 17:0 10-methyl, 10-methyl heptadecanoic acid.

sucrose, D-fructose, L-rhamnose, D-xylose, L-arabinose, D-mannose and L-lactic acid. Strain T16-1 was positive for cellulose, gelatin, skim milk and PLA degradation, nitrite from nitrate and starch hydrolysis. The temperature range for growth was 30 to 60°C. Strain T16-1 tolerates 3% NaCl in the medium (Table 2). Optimum temperature and pH, NaCl tolerant and utilization of mannitol, raffinose and arabinose of strain T16-1 as shown in Table 2 were different from type strain of *A. keratinilytica* WCC-2265^T (Puhl et al., 2009)

Cloning and sequencing of *pla*

The PCR product of 650 bp was ligated with pUC19/*Sma*I and transformed to *E. coli* JM109. Blue/white colony selection on LB+ampicillin+X-gal+IPTG plate was performed and white colony was selected as recombinant strain. The recombinant plasmid was extracted from the recombinant strain and cleaved by *EcoR*I and *Hind*III. The DNA fragment containing DNA sequences from plasmid pUC19 (vector) was obtained. Recombinant plasmid was extracted from recombinant strain and directly sequence based on the described method.

The DNA sequences of *pla* was translated to amino acid sequences, alignment (Figure 2) and blast searched for the most closely related gene from DDBJ/GenBank/EMBL amino acid sequence databases. The *pla* (222 amino acids) from *A. keratinilytica* T16-1 was the most closely related to *Streptomyces griseus* subsp. griseus NBRC 13350 putative secreted subtilisin-like serine protease of 43% identity and alkaline serine protease from *Streptomyces pristinaespiralis* ATCC 25486 of 46% identity which is different from the reported *pla* sequences.

DISCUSSION

The clear zone method is easy and significantly enhanced the isolation of PLA-degrading microorganisms from the environment. *Amycolatopsis* sp. strain HT-32 and strain K104-1 were isolated from soil samples by using plate count and clear zone method on an emulsified PLA agar plate for at least 14 days at 30°C (Pranamuda et al., 1997). In this work, clear zone formation was observed from the isolate T16-1 within 7 days on the plate at 50°C, indicating that the strain produced PLA-degrading enzyme at higher temperature. The maximum enzyme activity, 44.0 U/ml was obtained by using response surface methodology after 4 days cultivation in shake flask and 150 U/ml in an airlift fermenter at 0.5 vvm, initial pH 7.0 and 50°C (Sukkhum et al., 2009a).

In previous studies, several strains of *Amycolatopsis* sp. were reported as potent strains in their ability of PLA

Table 2. Comparison of phenotypic properties of strain T16-1 and WCC-2265^T.

Characteristic	T16-1	*WCC-2265^T
meso- **DAP type**	+	+
Conidia		
Chain arrangement	Flexous	Flexous-straight
Number in chains	10	5-15
Shape/ornamentation	Globose/spiny	Globose/aculeate
Color of colony		
ISP-2	Cream-yellow	Yellow-orange
ISP-3	Cream	Gray-white
ISP-4	Cream-yellow	Gray-white
Aerial hyphae		
ISP-2	Green-white	Trace
ISP-3	White	-
ISP-4	Greenish gray	-
Growth at/in		
Temperature	30-60℃, optimum at 50℃	30-55℃, optimum at 45℃
NaCl conc.	0-3% NaCl	>6% NaCl
pH	pH 6-8, optimum at pH 7	pH 4-10, optimum at pH 6-9
Utilization of		
Arabinose	+	+/-
Fructose	+	+
Glucose	+	+
Inositol	+	+
Mannitol	+	-
Raffinose	+	-
Rhamnose	+	+
Sucrose	+	+
Xylose	+	+

+, positive or present; -, negative or absent; +/-, not positive but not negative. *Puhl et al. (2009).

degradation (Pranamuda et al., 1997; Pranamuda et al., 2001; Tokiwa and Jarerat, 2004). Furthermore, other actinomycete strains such as *L. waywayandensis* and *K. aridum* also exhibited PLA degrading activity (Jarerat et al., 2004). Un-cultural strain such as *Paecilomyces*, *Thermomonospora* and *Thermopolyspora* were found to be predominant strains in compost that consist of PLA (Sangwan and Wu, 2008). Phenotypic and genetic data obtained from strain T16-1 lead to its identification as *A. keratinilytica*, which is a novel PLA-degrading thermophilic actinomycete strain.

Although, 16S rDNA sequencing and DNA-DNA hybridization indicated that strain T16-1 was the same as type strain (WCC-2265^T), some phenotypic characterizations, such as color of colony on ISP plates, optimum temperature and pH, NaCl tolerance and utilization of mannitol, raffinose and arabinose were different.

The gene sequencing demonstrated that *pla* is related to serine protease secreted from *Streptomyces griseus* subsp. *griseus*. This result correlates with the N-terminal of the purified enzyme which showed the similarity with serine protease from *Streptomyces avermitilis* MA-4680 as described by Sukkhum et al. (2009b).

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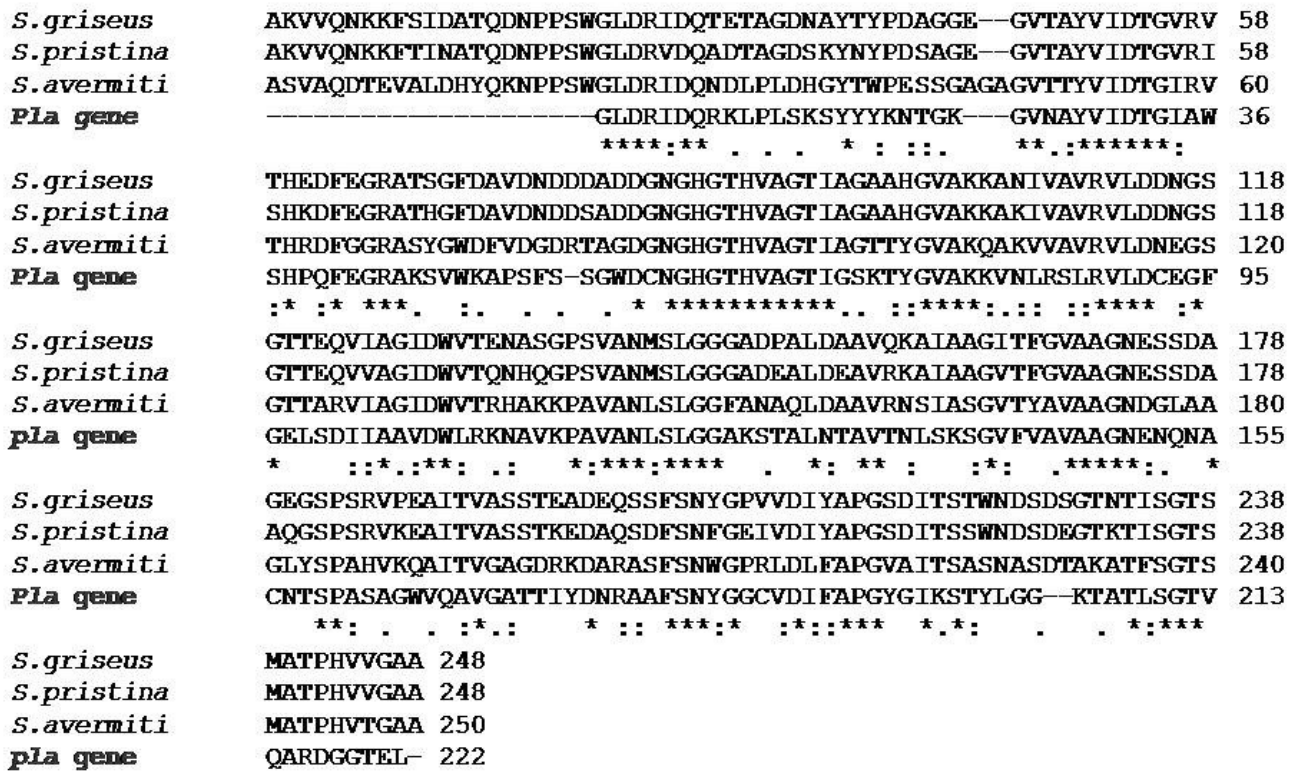

Figure 2. Alignment of *pla* with the most closely related amino acid sequences of serine protease.

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