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17-19 OCTOBER 2017

STT43 PROCEEDINGS BOOK

การประชุมวิชาการวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย ครั้งที่ 43 (วทท 43)
เข้าใจวิทยาศาสตร์ เข้าถึงเทคโนโลยี สร้างนวัตกรรม นำสังคมยั่งยืน

PROCEEDINGS BOOK

The 43rd Congress on Science and Technology of Thailand (STT43)

"Understand Science, Apply Technology, Create Innovation for Sustainable Society"

October 17-19, 2017
Venue: Chaloem Rajakumari 60 Building
Chulalongkorn University, Thailand

Organized by:
The Science Society of Thailand under the Patronage of His Majesty the King
In Association with
Chulalongkorn University

E_004_PF: ISOLATION AND CHARACTERIZATION OF POLYLACTIC ACID-DEGRADING BACTERIUM ISOLATED FROM WASTEWATER TREATMENT SLUDGE

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Abstract: This research isolated and screened polylactic acid (PLA)-degrading bacteria from wastewater treatment sludge and determined the levels of protease and PLA-degrading enzymes at various growth periods. Wastewater treatment sludges from six factories in Thailand were collected and spread on emulsified PLA agar plates. Selected bacterial isolates, based on the growth and clear zone formation on the emulsified PLA agar, were screened for protease production on skim milk agar plates. One isolate was found that produced a broad clear zone on skim milk agar and was designated as CH1. Isolate CH1 was identified as *Stenotrophomonas pavanii* by 16S rRNA gene sequencing. Measurements of enzyme production by *S. pavanii* CH1 in basal salt medium containing 0.2% (w/v) of PLA found that the highest levels of protease and PLA-degrading enzymes produced from *S. pavanii* CH1 were 355 U/mL at 24 h and 2.08 U/mL at 12 h of cultivation periods, respectively. It could be concluded that *S. pavanii* CH1 produces high levels of protease and PLA-degrading enzymes and could be further applied for PLA biodegradation.

Introduction: Petroleum-based plastics have widely used in the activities of daily life. Plastic waste is causing environmental problems because it is difficult to degrade and still accumulate in the environment. Nowadays, bioplastic is the interesting alternative plastic to replace petroleum-based plastics because it is produced from agricultural materials as a renewable resource and can be degraded by microorganisms under the proper environment. Polylactic acid (PLA) is one of bioplastics and synthesized from L-lactic acid which is mainly produced by fermentation process[1]. However, there are some limitations to treatment and disposal of bioplastic waste due to unable be recycled into new product and energy. Therefore, the proper treatment and disposal process of PLA waste need to be urgently studied.

The degradation process of PLA has involved the chemical hydrolysis and enzymatic degradation which possess PLA-degrading activity of microorganisms. However, PLA is more difficult to degrade than other biopolymers. Tokiwa and Jarerat (2003)[2] reported that the limited number of microorganisms were able to degrade PLA compared to other types of bioplastics. Enzymatic degradation of PLA occurs in soil and water by microorganisms which able to produce PLA-degrading enzymes. Several investigators reported that PLA is able to degrade by protease enzyme[3,4]. Therefore, this study focused on the isolation of PLA-degrading bacteria from wastewater treatment sludge and screening for protease production. Genus identification, growth pattern and levels of protease and PLA-degrading enzymes of a selected PLA-degrading bacterium were also studied.

Methodology:

Sample collection and isolation of PLA-degrading bacteria: Wastewater treatment sludges were collected from 6 factories in Thailand. Each sample was suspended in 0.85% sterile normal saline and made 10-fold serial dilution. Total viable bacterial cells in samples were enumerated on Nutrient agar (NA). Appropriate dilutions were spread on the emulsified PLA agar plates containing 0.1% (w/v) of PLA[5]. All plates were incubated at 30°C for 3-5 days. Growth and clear zone formation around bacterial colony on the emulsified PLA agar were considered to be the PLA-degrading bacteria. Appeared colony on the emulsified PLA agar plates were selected based on the difference of colony morphology.

Screening of PLA-degrading bacteria which are able to produce protease enzyme: Selected bacterial colonies were streaked on skim milk agar plates. Skim milk agar is used for identifying bacteria was produced protease from non-protease producing bacteria [6]. All plates were incubated at 30°C for 5 days. The clear zone formation around the bacterial colonies on skim milk agar was considered to be the

protease-producing bacteria[7]. The halo of clear zone was measured. Bacteria which exhibited the broader clear zone were selected for the further experiments.

Identification and growth determination of a selected PLA-degrading bacterium: Colony morphology of selected bacterial isolate was observed on NA plates after incubation for 24 h. Bacterial isolate was determined the Gram by Gram staining and observed cell morphology using Scanning Electron Microscope (SEM). Genus and species was identified by sequencing analysis of 16S rRNA gene. Growth curve of selected isolate was determined by culturing in Nutrient broth (NB) with continuous shaking at 150 rpm at 2, 4, 6, 8, 12, 24, 36, 48, 72 and 96 h, respectively. Bacterial growth was monitored using spectrophotometer by measuring at wavelength 600 nm (OD_{600}).

Quantitative determination of protease and PLA-degrading enzymes: Selected bacterial isolate was inoculated in basal salt medium (BSM) containing 0.2% (w/v) of PLA. Culture flask was incubated at 30°C with shaking at 150 rpm for 96 h. The cell-free supernatant as a crude enzyme was collected by centrifugation at 2, 4, 6, 8, 12, 24, 36, 48, 72 and 96 h, respectively. Protease activity in the cell-free supernatant was examined using casein as the substrate. According to the method of Brock et al. (1982)[8], an assay of the protease activity was performed using the azocasein method. The assay of PLA-degrading enzyme was conducted according to the method of Pranamuda et al. (2001)[9]. One unit (U) of PLA-degrading activity was defined as the amount of enzyme required to produce 1 mol of L-lactic acid in 1 min. Lactic acid formed during the enzymatic reaction was analyzed according to the method of Taylor (1995)[10].

Results and Discussion:

Isolation of PLA-degrading bacteria: Samples of wastewater treatment sludge were isolated on the emulsified PLA agar plates containing 0.1% (w/v) of PLA. The viable cells in wastewater treatment sludges were also enumerated. On the emulsified PLA agar plates, the colonies surrounded by clear zone with difference colony morphology were picked up and patched on the new emulsified PLA agar plates to ensure the forming of the clear zone. The results of the number of viable bacteria and number of selected PLA-degrading isolates present in Table 1.

The number of bacteria in wastewater treatment sludge was approximately 10^6-10^7 CFU/g sludge. The highest number of viable bacteria was found in the wastewater treatment sludges from Tuna processing and dairy factories. A total of 18 isolates which were selected based on the difference of colony morphology and formation of clear zone on the emulsified PLA agar were selected. It indicates that these isolated can use PLA as a carbon source for their growth. Apinya et al. (2015)[11] reported *Pseudonocardia* sp. RM423 used PLA as carbon source and was able to grow on emulsified PLA agar.

Table 1. The number of viable bacteria and PLA-degrading bacteria isolated from wastewater treatment sludges

Sources of wastewater treatment sludge	Number of viable bacteria (CFU/g)	Number of selected PLA-degrading bacteria (Isolates)
- Tuna processing factory	1.1×10^7	2
- Rice vermicelli factory #1	2.0×10^6	1
- Rice vermicelli factory #2	3.3×10^7	3
- Plastic factory	2.2×10^6	4
- Coconut milk factory	7.0×10^5	3
- Dairy factory	1.1×10^7	5
Total	-	18

Screening of PLA-degrading bacteria which are able to produce protease enzyme: All 18 isolates were re-streaked on NA agar to ensure the pure culture. They were screened for protease production by streaking on the skim milk agar. A qualitative screening for the proteolytic activity of the isolates was indicated by growth and clear zones appearance on skim milk agar. Two isolates, designated as CH1 and UT7 showed the growth and clear zone formation on the skim milk agar (Table 2). CH1 and UT7 were isolated from wastewater treatment sludge of rice vermicelli factory #2 and wastewater treatment sludge of plastic factory, respectively.

Table 2. Number of protease-producing bacteria screened on skim milk agar

Clear zone formation	Isolate (%)
-	16 (88.9%)
+	2 (11.1%)
Total	18 (100%)

Ponmurugan (2007)[12] reported that several strains of *Bacillus* spp. were able to hydrolyse proteins due to their protease activity. Ponmurugan (2007)[12] isolated *Bacillus* spp. from egg granules and grape juices which were designated as EB-2 and JGB, respectively. The sizes of halo zone around colonies of isolates EB-2 and JGB on skim milk agar plates were 32.3 and 25.7 mm, respectively.

To qualitative determination of protease production, a 10 µL of inoculum of CH1 and UT7 was dropped on the surface of skim milk agar plates and incubated at 30°C for 3 days. The size of clear zone around the colony on skim milk agar was measured. The results found that the diameters of the clear zone of CH1 and UT7 were 26.10 and 54.0 mm, respectively. In addition, the ratio of the diameter of the clear zone and diameter of colony on skim milk agar was calculated. The results found that CH1 and UT7 had the size of the clear zone around the colony by 8.6 and 2.85 mm, respectively. It indicated that CH1 was able to hydrolyse skim milk protein better than that of isolate UT7. Therefore, CH1 was selected for use in the further experiments.

Identification of a selected PLA-degrading bacterium: CH1 was identified by sequencing analysis of 16S rRNA gene and analyzed by BLASTn program from GenBank database.The results found that sequences of 16S rRNA gene of isolate CH1 had 99.81 % similarity with *Stenotrophomonas pavanii* DSM25135. *Stenotrophomonas pavanii* sp. nov. is a nitrogen-fixing bacterium which was isolated from stems of a Brazilian sugarcane[13]. *S. pavanii* CH1 was a Gram-negative and rod-shaped bacterium. Colony morphology of *S. pavanii* CH1 was observed on NA plate after incubation at 30°C for 24 h (Figure 1a) and cell morphology was observed under SEM presents in Figure 1b.

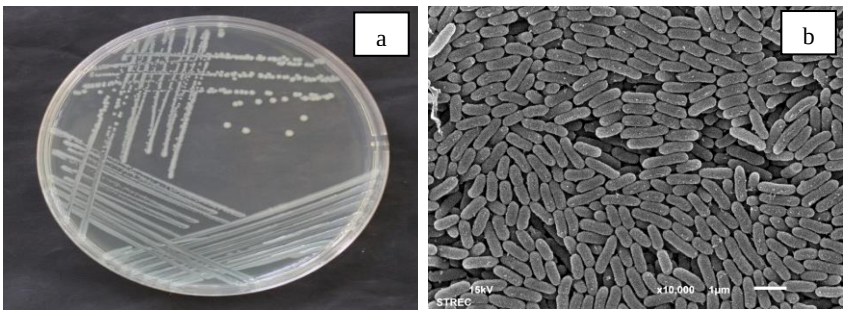

Figure 1. (a) Colony morphology and (b) cell morphology under Scanning Electron Microscope at 10000× magnification of *S. pavanii* CH1

Quantitative determination of protease and PLA-degrading enzymes at various growth periods: The growth and the levels of protease and PLA-degrading enzymes produced from of *S. pavanii* CH1 were determined by culturing in BSM containing 0.2% (w/v) of PLA for 96 h. The results are presented in Figure 2 and 3.

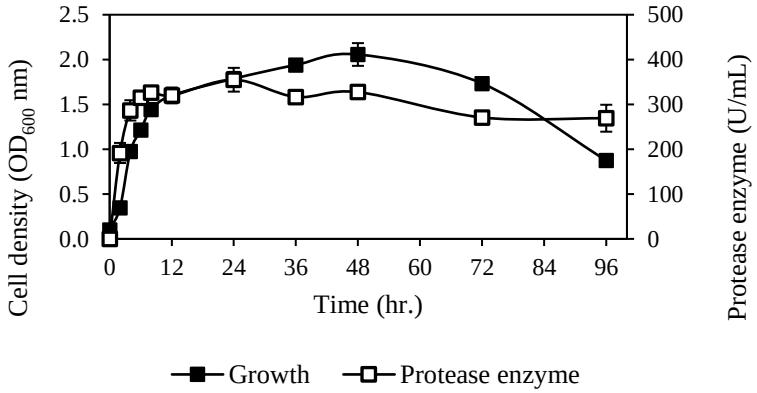

Figure 2. Growth and protease production of *S. pavanii* CH1

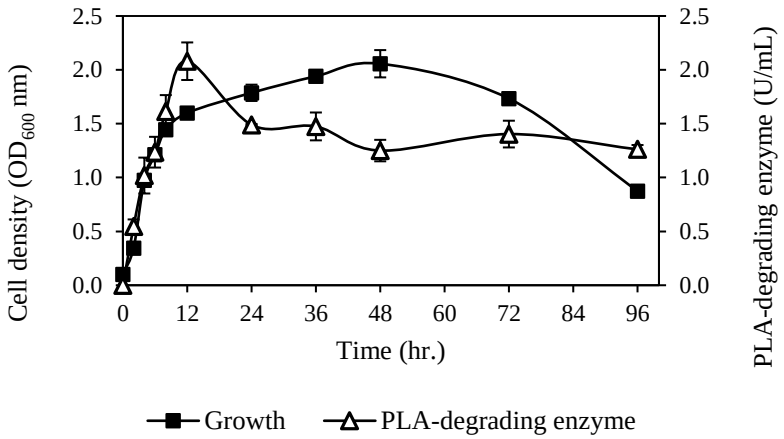

Figure 3. Growth and PLA-degrading enzyme production of *S. pavanii* CH1

The results in Figure 2 found that protease production corresponded with the bacterial growth and reached a maximum level (355.0±26.52 U/mL) at the stationary phase (24 h). In addition, the highest level of PLA-degrading enzyme was 2.08±0.17 U/mL at 12 h of cultivation period (Figure 3). Our findings corresponded with the study of Patel et al. (2005)[14], who reported that halo-alkaliphilic *Bacillus* sp. produced the maximum level of protease by 397 U/mL. Sukkhum et al. (2012)[15] reported that *Actinomadura keratinilytica* T16-1 which was cultured in the medium containing yeast extract as the organic nitrogen source produced PLA-degrading enzyme by 22 U/mL. Jarerat et al. (2004)[16] found that the maximum levels of PLA-degrading activity produced from *Lentzea waywayandensis* and *Amycolatopsis oriental* in basal medium supplemented with gelatin were 118 and 40 U/mL, respectively. The role of gelatin is an inducer for enhancing the production of PLA-degrading enzyme[16]. Our results indicate that *S. pavanii* CH1 was able to produce protease and PLA-degrading enzymes.

Conclusion: CH1, a PLA-degrading bacterium isolated from wastewater treatment sludge from rice vermicelli factory was able to grow on the emulsified PLA agar and had the highest the size of clear zone around colony on skim milk agar. It was identified as *S. pavanii*, a Gram-negative and rod-shaped bacterium. It produced the maximum levels of protease and PLA-degrading enzymes by 355.0 U/mL at 24 h and 2.08 U/mL at 12 h of cultivation periods, respectively. Due to the production of protease and PLA-degrading enzyme, the effects of enzymatic inducers e.g., gelatin on the production of these enzymes by *S. pavanii* CH1 should be further investigated.

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Acknowledgements: This research is supported by Thailand Research Fund (TRF) under the Directed Basic Research Grant and Faculty of Environment and Resource Studies of Mahidol University Alumni Association.