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# Isolation and characterization of PLA-degrading bacteria from landfill soil at mesophilic temperature

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**Abstract.** This study aimed to isolate and characterize polylactic acid (PLA) degrading bacteria present in landfill soils located in Pekan, Pahang. The conducted study involves collecting four soil samples from four different sources and stored at room temperature before undergoing serial dilution from dilution factor (DF)  $10^{-1}$  until dilution factor  $10^{-10}$ . Characterization of bacteria via Gram staining was done only on isolates (69 colony) with DF 10-6 until DF 10-10 but only 13 isolates from plate with DF 10-8 and 10-10 were re-streaked to obtain single colony. The single colony obtained were further characterized for their morphological characteristics and tested for presence of protease enzyme by streaking the colony on skim milk agar which resulted with only four (A10K, A10B, B8A and D10A) showing positive presence of protease enzyme. The four plates were tested for catalase using 3%(v/v) hydrogen peroxide and all resulted with vigorous bubbling which indicate positive catalase enzyme. Relative enzyme activity was calculated for all four bacteria through observation of halo zones on skim milk agar, and isolate A10B shows the highest relative enzyme activity with 3.86, and thus showing potential to accelerate the degradation of PLA at mesophilic temperature. Therefore, in this study, it is proven to be possible to isolate bacteria that can degrade PLA at mesophilic temperature from landfill soil.

## 1. Introduction

Poly(lactic acid) (PLA) is an aliphatic polyester and it is one of the biodegradable plastics commercially available worldwide. It was first discovered by Carothers (at DuPont) in 1932 where he was able to produce a low molecular weight PLA through heating of lactic acid under vacuum while removing the condensed water. The restriction of the production at that time was the inability to increase the molecular weight of the product; and finally, via ring-opening polymerization of the lactic acid only was high molecular PLA was synthesized [1]. PLA is greatly favoured due to its excellent mechanical properties, easy processability, biocompatibility and biodegradability [2]. Furthermore, PLA has found numerous applications in diverse fields such as medical, agriculture, textile, and packaging due to its versatility in properties [3].

PLA shows a promising future to be an alternative for petroleum-based plastics because its monomer may be produced from non-toxic renewable feedstock such as cassava, corn, starch and sugarcane [4].



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In comparison with other biopolymer, the production of PLA is considerably advantageous in many aspects such as: (a) production of lactide monomer through fermentation can be obtained from a renewable agricultural source; (b) saves a significant amount of energy; (c) the capabilities to recycle the product back to lactic acid via hydrolysis or alcoholysis; (d) reduction of landfill wastes; (e) improvement in agricultural sectors; and (f) the ability to adapt a physical properties through material modification [5]. Due to the similarity of material properties with synthetic thermoplastics, PLA has made a breakthrough in the market as a packaging thermoplastic for general packaging applications [6, 7].

According to a study [8], the chemical hydrolysis acts as the main depolymerisation process that occurs during PLA degradation and it became a limiting factor for the microorganisms to biodegrade the PLA. Since the PLA can be degraded in both aerobic and anaerobic conditions, the chemical hydrolysis occurs more rapidly at elevated temperature especially under thermophilic conditions [9]. This procure the problem that PLA faces in the context of environmental degradation in which when a PLA-based product is discarded in the environment to degrade, the time of degradation to occur is longer compared to other bioplastic such as polyhydroxyalkanoates (PHA) or polyethylene glycol (PEG) [10]. This is because, PLA is greatly resistant towards the attack of microorganisms in soil or sewage at mesophilic conditions and is primarily temperature-and-pressure dependant [11]. In addition, most PLA-degrading microorganisms in the environment are not widely distributed compared to microorganisms that associate with other type of biodegradable plastics [12, 13].

In the previous work, the study of PLA isolation and characterization from landfill soils were done at thermophilic temperature which is less efficient due to the requirement of more energy to elevate the surrounding soil before degradation by microorganism can occur [14]. In the study of [15] found in their study that the strain, identified as *Bacillus brevis*, was observe to assimilate and degrade PLA at an optimum temperature of 60°C. A later study by [16] shows the degradation of PLA by the first thermophile belonging to the genus *Geobacillus*. A more recent study by [17], shows the physical and chemical changes of PLA sheets during burying test at stimulated landfill condition at 60°C. The condition enhances PLA degradation up to 90% of weight loss in 90 days. Moreover, the previous studies by [18] and [19] reported the low biodegradability on anaerobic conditions at 30°C - 37°C. The lack of studies regarding PLA-degrading mesophiles raises the need to further this expand this territory.

Therefore, this paper presents the study of isolating and characterizing bacteria that can degrade PLA at mesophilic temperature with the presence of protease enzyme. In this study, four soil samples from four different sources were collected and stored at room temperature before undergoing serial dilution from dilution factor (DF)  $10^{-1}$  until dilution factor  $10^{-10}$ . Characterization of bacteria via Gram staining was done only on isolates (69 colony) with DF  $10^{-6}$  until DF  $10^{-10}$  but only 13 isolates from plate with DF  $10^{-8}$  and  $10^{-10}$  were re-streaked to obtain single colony. The single colony obtained were further characterized for their morphological characteristics and tested for presence of protease enzyme by streaking the colony on skim milk agar. Chosen microbe isolates were tested for catalase using 3%(v/v) hydrogen peroxide and the relative enzyme activity was calculated for all four bacteria through observation of halo zones formation.

## 2. Materials and method

### 2.1. Materials

All chemicals, including ethanol, PLA powder, dichloromethane, Tween80, nutrient agar, BSM broth, gram staining chemical kit, skim milk powder, hydrogen peroxide, and ¼ strength Ringer's tablet, were bought from Sigma-Aldrich and equipment were provided by Universiti Malaysia Pahang.

### 2.2. Soil sample collection

Soil samples were obtained from four sources to broaden the possibility of finding mesophilic bacteria at other places beside the targeted landfill soil. In this study, two samples were taken from within a landfill in Pekan, Pahang (A, soil attached to plastic waste in landfill; B, soil on the ground of the

landfill), while one sample was obtained from outside the landfill (C, soil from UMP ground), and one sample was obtained from a provided compost (D, soil in UMP compost).

### 2.3. PLA-emulsified medium preparation

PLA-emulsified medium was prepared by dissolving PLA powder (0.1% w/v) in 50mL dichloromethane solution. Mixture was then sonicated in distilled water and was added with Tween80 (1% v/v) for 1 hour. After sonication, dichloromethane was removed through heating in a fume hood at temperature 49°C. The medium was autoclaved at 121°C and 15psi for 20 minutes and poured into nutrient agar mixtures via pouring technique.

### 2.4. Isolation of PLA-degrading bacteria

The isolation process was conducted after soil samples underwent serial dilution until dilution factor (DF)  $10^{-10}$ . Diluted samples were transferred onto the PLA-emulsified medium via pour plate technique and incubated at 30°C in the incubator for 3-5 days until bacterial formation was visible on the plate. Yellow zone indicates PLA degradation activity carried out by PLA degrader [20]. Plates containing bacteria from DF  $10^{-6}$  until DF  $10^{-10}$  were taken to be re-streaked. Each microbe with different visuals was transferred onto new PLA-emulsified agar and purified via streak-plate technique until single colony is obtained. The number of isolates found in soil samples can be observed in Table 1. The single colonies from plates with DF  $10^{-8}$  and  $10^{-10}$  was further re-streaked based on the justification that the microorganisms present shows distinct physical characteristics and is the most accessible to pick up microbes. During the process of isolation, only single colony of 13 isolates were obtained through continuous streaking (A8Z, A8K, A8A, B8K, B8Z, B8A, C8K, C8C, D8K, D8B, A10K, A10B, D10A). The physical shape of the remaining isolates remains dilated even after a series of streaking which may be due to long incubation of period. According to [21], different microorganisms have different incubation hour and the extended time of incubation will cause the colony to dilate.

**Table 1.** Number of isolates found in soil samples

Dilution Factor (DF)	Number of isolates from soil sample			
	A	B	C	D
$10^{-6}$	18	15	24	69
$10^{-7}$	6	3	8	12
$10^{-8}$	5	3	3	4
$10^{-9}$	3	2	3	3
$10^{-10}$	2	-	1	1

### 2.5. Characterization of PLA-degrading bacteria

**2.5.1. Morphological Study and Gram Staining.** Morphological study is the most common identification criteria that was used to characterize the bacterial growth and were done by observing three main elements of the microbes which were the form, elevation, and margin of the isolates. [22] was referenced to determine these three elements observed in the samples.

Gram staining characterization was done by taking a thin smear of bacterial isolates was separately made on clean glass slide. The smear was fixed by rapidly passed through the flame of a Bunsen burner three times. Then, the smear was stained by crystal violet for one minute and rapidly washed with water followed by flooded with gram's iodine. After one minute, the slide was washed again with water and decolorized with alcohol. The smear was washed immediately with water and covered with safranin for one minute. The slide was washed and air dried, and finally observed under microscope. The gram-negative organisms were stained pink and the gram-positive organisms was dark violet in colour. Mix isolate (mixture of gram negative and gram positive) obtained was be sub-cultured again and again until pure isolate was obtained.

*2.5.2. Qualitative screening of protease presence.* The purified colonies were tested for protease presence by streaking the single colonies on the skim milk agar and incubated at 30°C for 3 days. Each colony was observed for formation of clear zones which proves the presence of protease enzyme which can degrade PLA. PLA-degrading bacteria that exhibit the broadest clear halo zones were selected for further experimentation [23].

*2.5.3. Catalase Test.* Hydrogen peroxide (3%, v/v) was prepared for the test. Catalase test was conducted by using 0.2mL of hydrogen peroxide solution placed on a clean glass slide. Bacterial colony was picked using sterile toothpick and rubbed on glass slide. The presence of vigorous bubbling shows within 10 – 15 seconds, means the test were positive in result.

*2.5.4. Quantitative analysis of relative enzyme activity.* For the qualitative analysis of relative enzyme activity, equation (1) was used to calculate the results. The isolates were each streaked onto a new plate of skim milk agar and incubated at 30°C for 48 hours. After the incubation, the diameter of the colony and clear zones formed was measured and recorded, before calculating its relative enzyme activity.

$$\text{Relative enzyme activity} = \frac{\text{diameter of halo zone} - \text{diameter of colony}}{\text{diameter of colony}} \quad (1)$$

### 3. Result and discussion

#### 3.1. Morphological study and Gram staining characterization of isolates

The result of the form of bacterial colony was obtained and it showed circular form and punctiform were most identified with five colonies each, followed by filamentous form identified on two colonies, and one colony was identified as irregular form. There was no identification of rhizoid form, and spindle form among the isolates. For the identification of colony elevation, result shows that convex and flat was most identified among the colonies with five colonies each. Two colonies were identified to have a raised elevation while only one was identified as pulvinate. No colonies were identified as umbonate. The identification of bacterial margin showed that nine colonies were identified as an entire margin, followed by two curled margin, one undulate margin, and one filamentous margin. The result was recorded in Table 2.

**Table 2.** Morphological study.

Isolation		Morphological characteristics		
DF	Isolate Code	Form	Elevation	Margin
10 <sup>-8</sup>	A8Z	Filamentous	Raised	Curled
	A8K	Punctiform	Convex	Entire
	A8A	Circular	Flat	Entire
	B8K	Irregular	Convex	Filamentous
	B8Z	Punctiform	Convex	Entire
	B8A	Punctiform	Flat	Curled
	C8K	Circular	Flat	Entire
	C8C	Circular	Pulvinate	Undulate
	D8K	Punctiform	Flat	Entire
	D8B	Circular	Raised	Entire
10 <sup>-10</sup>	A10K	Punctiform	Convex	Entire
	A10B	Circular	Flat	Entire
	D10A	Filamentous	Convex	Entire

Based on the results of the Gram staining of this study, it was observed that among the 13 isolates, nine out of it shows gram positive colour while the remaining four were gram negative. Eight out of nine gram positive bacteria exhibit rod-like shape which indicates that it is bacilli, while one was cocci.

For gram negative, two out of four were coccobacilli, one was bacilli, and another one was cocci. The result was recorded in Table 3 and the variation of Gram staining results can be seen in Figure 1.

**Table 3.** Gram staining results for each of the isolates.

Isolate	Gram Staining	Bacterial Shape	Pure/Mix
A8Z	Positive	Bacilli	Pure
A8K	Positive	Bacilli	Pure
A8A	Positive	Cocci	Pure
B8K	Negative	Coccobacilli	Pure
B8Z	Negative	Cocci	Pure
B8A	Positive	Bacilli	Pure
C8K	Positive	Bacilli	Pure
C8C	Negative	Coccobacilli	Pure
D8K	Negative	Bacilli	Pure
D8B	Positive	Bacilli	Pure
A10K	Positive	Bacilli	Pure
A10B	Positive	Bacilli	Pure
D10A	Positive	Bacilli	Pure

### 3.2. Qualitative screening for presence of protease

In qualitative screening of PLA-degrading bacteria, all 13 isolates were evaluated for the detection of protease enzyme by observing and evaluating the clear zone formation on skim milk agar. A single colony of each isolate were streaked on skim milk agar and incubated at 30°C for 72 hours. Results can be observed in Table 4, in which out of 13 isolates only 4 were able to form clear zones on skim milk agar, indicating the presence of protease enzyme by the bacteria. Based on the study, isolates from plate A10K, A10B, B8A, and D10A.

**Table 4.** Presence of halo zone on skim milk agar.

Isolate	Presence of halo zone
A8Z	No
A8K	No
A8A	No
B8K	No
B8Z	No
B8A	Yes
C8K	No
C8C	No
D8K	No
D8B	No
A10K	Yes
A10B	Yes
D10A	Yes

### 3.3. Catalase test

The four bacterial colony that shows positive protease enzyme presence were further tested for catalase test. Using approximately 0.2mL of hydrogen peroxide (3%; v/v) on a clean glass slide, catalase test was performed by rubbing the colony on the glass slide using a sterile toothpick. All four colony shows vigorous bubbling within 10-15 seconds. This conclude that all four bacteria colony have catalase enzyme.

### 3.4. Regression model for protease activity

For the qualitative analysis of relative enzyme activity, equation (1) was used to calculate the results. The isolates were each streaked onto a new plate of skim milk agar and incubated at 30°C for 72 hours. After the incubation, the diameter of the colony and clear zones formed was measured and recorded, before calculating its relative enzyme activity. Relative enzyme activities results were shown in Table 5.

The relative enzyme activity analysis is a gross measure of enzyme production and enzyme activity. Protease activity was visualized by the formation of clear zone when a certain single colony is streak anew. The measurement itself is highly dependent on the type of organisms, the media used, and the incubation time as different factor will give different result. The diameter of each colony and activity zone was measured in two dimensions at 90° of each other and the values averaged. Individual colony differs only by no more than 5% from the overall average [24].

**Table 5.** Relative enzyme activity for each isolate.

Isolates	A10K	A10B	B8A	D10A
Colony diameter (cm)	1.0	0.7	0.9	1.2
Halo zone diameter (cm)	1.6	3.4	2.1	2.3
Relative enzyme activity	0.60	3.86	1.33	0.95

## 4. Conclusion

Overall, PLA-degrading bacteria were successfully isolated from landfill soil. After serial dilution, only plate with DF  $10^{-8}$  and  $10^{-10}$  were taken for further studies due to the microorganisms showing consistent, and distinctive physical characteristics and most accessible to microbes which resulted with 13 isolates. These isolates were firstly characterized through observation of their morphological form, elevation and margin, and then tested with Gram staining. The result shows 9 out of 13 were Gram positive mostly with bacilli shape. Out of the 13 isolates, four isolates (A10K, A10B, B8A, and D10A) show the presence of protease enzyme due to halo zone formation in skim milk agar. Further catalase testing shows that all isolates have positive results. The relative enzyme activity of the four isolates were obtain through streaking of single colony on skim milk agar and observing the halo zone formation for each isolate. The result shows that A10B have the highest relative enzyme activity and have a high potential to produce more protease enzyme. The presence of protease from the bacteria found in landfill soil at mesophilic temperature has a high potential to be exploited in both the microbiology and biotechnology fields. The future work of the present study may focus on identifying the bacterial strain (DNA identification) and optimization of enzyme in order to gain higher activity by manipulating specific condition beside temperature.

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